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The *Drosophila retained/dead ringer* gene and ARID gene family function during development

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ABSTRACT The recently discovered ARID family of proteins interact with DNA through a phylogenetically conserved sequence termed the A/T Interaction Domain (ARID). The *retained/dead ringer (retn/dri)* gene of *Drosophila melanogaster* is a founding member of the ARID gene family, and of the eARID subfamily. This subfamily exhibits an extended region of sequence similarity beyond the core ARID motif and a separate conserved domain termed the REKLES domain. *retn/dri* is involved in a range of developmental processes, including axis patterning and muscle development. The *retn/dri* ARID motif has been shown by *in vitro* studies to exhibit sequence-specific DNA binding activity. Here we demonstrate that the ARID domain is essential for the *in vivo* function of *retn/dri* during embryonic development by showing that a mutant form of RETN/DRI, deleted for part of the ARID domain and unable to bind DNA *in vitro*, cannot rescue the *retn/dri* mutant phenotype. In the presence of wild-type RETN/DRI this construct acts as a dominant negative, providing additional support for the proposal that RETN/DRI acts in a multiprotein complex. In contrast, we are yet to find an *in vivo* role for the REKLES domain, despite its clear evolutionary conservation. Finally, we have used germline clone analysis to reveal a requirement for *retn/dri* in the *Drosophila* preblastoderm syncytial mitoses.

KEY WORDS: *ARID motif, gene regulation, cell cycle control, DNA binding proteins*

Introduction

The development of multicellular organisms requires tight spatial and temporal control of switches in the activity of key regulatory genes. Tissues are specified by transcriptional regulatory cascades that respond to a variety of extracellular and intracellular cues. It has become clear in recent years that the regulators of tissue-specific gene expression, the transcription factors, are assembled into multi-protein complexes. These complexes include DNA-binding proteins, which recruit other components that are not able to bind DNA directly. The composition of complexes assembled on a specific promoter can be transient, as seen in the cascade of gene regulation in the early *Drosophila* embryo. Curiously, many transcription factors fall into a relatively small number of phylogenetically conserved families, although different family members can assume very different developmental roles in different organisms (see, for example, Lall and Patel, 2001). As a result, studies of members of conserved protein families very often provide new approaches to understanding mechanisms that regulate a variety of developmental events.

One of the most recent of the phylogenetically conserved families to have been identified is the ARID family (see Kortschak *et al.*, 2000). The family was discovered simultaneously by the characterisation of two proteins, mouse Bright (Herrscher *et al.*, 1995; reviewed in Webb, 2001) and *Drosophila* Dead ringer (Gregory *et al.*, 1996). The *dead ringer* gene has since been shown to correspond to the *retained* (*retn*) gene (Schüpbach and Weischaus, 1991; Ditch, Pitman, Finley, Edeen and McKeown, unpublished observations) and is here referred to as *retn/dri*. Bright and RETN/DRI were found to contain a conserved sequence-specific DNA binding motif, termed the A/T Interaction Domain (ARID) (Herrscher *et al.*, 1995; Gregory *et al.*, 1996). Members of this family have since been found in *Saccharomyces cerevisiae, Caenorhabditis elegans, Danio rerio,* mouse and human (Fig. 1A, Kortschak *et al.*, 2000). Sequence-specific DNA binding appears not to be an intrinsic feature of the ARID motif, since the ARID motif proteins human SMARCF1 and *Drosophila* OSA (orthologs of yeast SWI1) exhibit non-sequence-specific DNA binding (Dallas *et al.*, 2000; Collins *et al.*, 1999).

More detailed sequence comparisons revealed that the ARID family proteins could be divided into three subfamilies. The first of

Abbreviations used in this paper: ARID, AIT-rich interaction domain; EMS, ethylmethanesulphonate.

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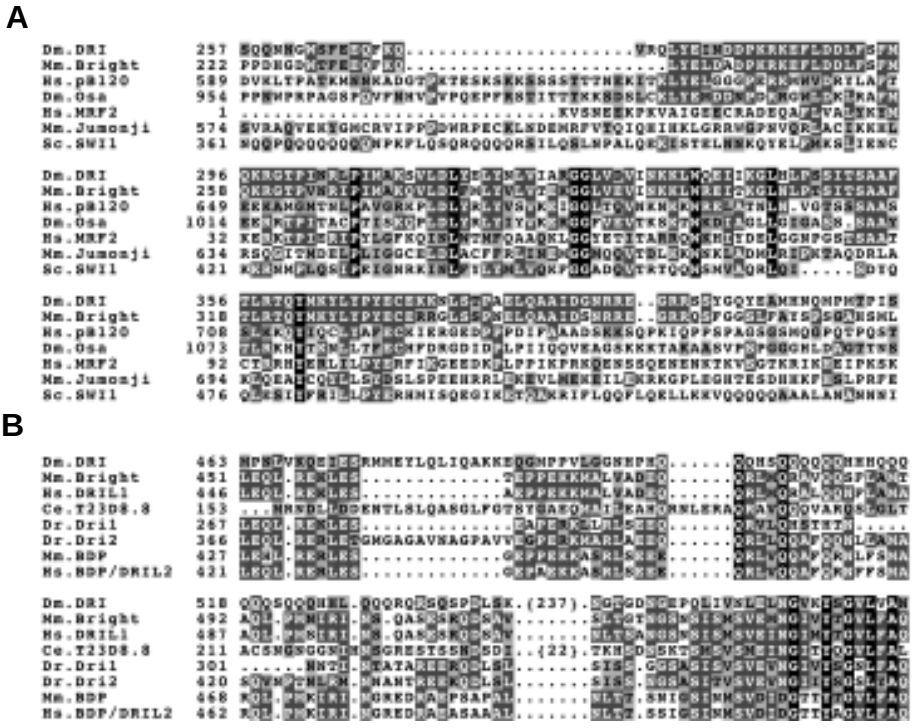

Fig. 1. Sequence comparison of the ARID and REKLES motifs of selected ARID family proteins. **(A)** *eARID and ARID alignment showing the extended region of homology of RETN/DRI and Bright, both N and C-terminal to the core ARID which is conserved across all family members.* **(B)** *Alignment of amino acid sequences of the REKLES region from all known eARID proteins.*

these, which contains *Drosophila* RETN/DRI and mouse Bright, exhibits an extended region of similarity either side of the core ARID motif. We have termed this larger region of similarity the extended ARID (eARID) motif (Kortschak *et al.,* 2000). eARID subfamily members also share an independent conserved domain termed the REKLES domain (see below). The second subfamily is defined on the basis of the presence of several C-terminally located conserved domains, including PHD finger domains and a highly conserved PLU-domain. This sub-family includes *Ustilago maydis* Rum1 (Quadbeck-Seeger *et al.*, 2000), mouse and human SmcX/Y (Agulnik *et al.,* 1994a; Agulnik *et al.,* 1994b), mouse Jumonji and Desrt (Takeuchi *et al.*, 1995; Lahoud *et al.*, 2001), and human PLU-1, XE169, RBP1, RBP2, MRF1 and MRF2 (Lu *et al.*, 1999; Wu *et al.*, 1994; Fattaey *et al.*, 1993; Huang *et al.*, 1996). The third subfamily of ARID proteins includes yeast SWI1 (Quinn *et al.*, 1996), *Drosophila* OSA/ELD (Treisman *et al.*, 1997), a *Caenorhabditis elegans* ortholog (GeneBank accession no.U80439), mouse Osa1 and human SMARCF1/OSA1/p270/B120/BAF250 (Takeuchi *et al.*, 1997; Kozmik *et al.*, 2001) proteins. In addition to the ARID motif, these proteins have N-terminal Osa Homology Domains 1 and 2 (OHD1, OHD2) containing four LXXLL motifs, which are implicated in nuclear hormone receptor binding (NR-box).

From the outset, it appeared that members of this gene family would play roles in developmental regulation. For example, *retn/dri* exhibits a highly regulated temporal and spatial pattern of expression (Fig. 2, Gregory *et al.*, 1996; Shandala *et al.*, 1999), while Bright, a B cell regulator of IgH transcription also exhibits tissue-restricted patterns of expression (Herrscher *et al.*,1995; Webb *et al.*, 1998).

Evidence for a developmental role for ARID family genes has also come from genetic analysis. The *SWI1* gene of *Saccharomyces cerevisiae* is part of the Swi/Snf complex required for the regulation of many genes involved in simple developmental processes such as mating type switching (see Sudarsanam and Winston, 2000, for a review). More recently, the *regulator U. maydis* 1 (*rum1*) gene has been shown to be essential for normal development of *Ustilago maydis* spores (Quadbeck-Seeger *et al.*, 2000). The effect on sporulation appears to be mediated by downregulation of a very defined set of genes, including *egl1, dik1, lga2, hum2*, that are normally induced by two homeobox genes, *bE* and *bW*, encoded by the *U. maydis* mating type locus.

A role for the *Drosophila retn/dri* gene in embryonic development was revealed by characterisation of *retn/dri* EMS-induced alleles (Shandala *et al.*, 1999). *retn/dri* was found to be essential for several aspects of embryogenesis, including anterior/posterior and dorsal/ventral patterning. One of the targets of *retn/dri* transcriptional regulation in the blastoderm embryo appears to be the dorsal-specific gene *zerknüllt* (*zen*). *zen* is normally expressed in a narrow dorsally located stripe along the embryo. In *retn/dri* mutant embryos, *zen* expression fails to refine to its normal amnioserosa-specific expression (Valentine *et al.*, 1998). The activity of RETN/DRI in this case is to convert the normal transcriptional activator Dorsal (DL) into a repressor by binding adjacent to DL and creating a surface to which the Groucho (GRO) WD-40 repeat co-repressor protein binds (Valentine *et al.*, 1998). GRO has been found to interact with the N-terminal tail of histone H3 and with the histone deacetylase Rpd3, an enzyme involved in chromatin condensation and gene inactivation (Palaparti *et al.*, 1997; Chen *et al.*, 1999).

Repression of *huckebein* along the ventral side of the embryo trunk is also mediated by RETN/DRI and DL, through recruitment of GRO to the ventral repression element of *hkb* (Hader *et al.*, 2000). *huckebein* is a *Drosophila* terminal gap gene expressed in the anterior and posterior caps. It is activated along the ventral side of the embryo by DL. For both *zen* and *hkb*, there is a difference between the behaviour of the endogenous genes and of the minimal repression element derived from them, the minimal elements exhibiting much greater sensitivity to *retn/dri* regulation. This is presumably the result of a level of redundancy in the repression of the wild-type gene that is lost in the reduction to the minimal regulatory element.

In addition to its role as a repressor, evidence suggests that RETN/DRI can act as an activator. *retn/dri* is required for activation of the *argos* gene in two near-terminal domains of expression in the blastoderm embryo, accounting for the head skeleton phenotype observed in *retn/dri* mutant embryos (Shandala *et al.*, 1999).

None of these studies addressed the significance of the various RETN/DRI motifs, including the ARID motif, in the *in vivo* function

of the protein. This issue is made more significant by the observation that the ARID motif is dispensable for the *in vivo* function of the *S. cerevisiae* SWI1 protein (C. Peterson, personal communication). Here we provide evidence that the ARID domain is essential for the *in vivo* function of *retn/dri*. In addition, we explore the function of the separate REKLES domain within *retn/dri*. Finally, we show that *retn/dri* is required for normal mitosis during the syncytial cleavage stage of *Drosophila* development.

Results

The ARID Domain is Necessary for DNA Binding

To assess the importance of the RETN/DRI ARID motif, *in vitro* mutagenesis was used to generate a *retn/dri* construct, termed ARIDδH5, in which the third α-helix of the ARID motif was mutated. This helix was selected on the basis that it is highly conserved and includes an invariant tryptophan residue found in all ARID domains, indicating that it should be essential for ARID function. The RETN/DRI eARID, spanning the sequence from amino acid 258 to 410, has previously been shown to be sufficient for sequence-specific DNA binding to an oligonucleotide consisting of repeats of a consensus Engrailed binding site termed NP (Gregory *et al.*, 1996). To determine whether helix 5 of the RETN/DRI ARID is necessary for binding to the NP sequence, electrophoretic mobility shift assays were conducted using both the wild-type ARID (GST-RETN/DRI$_{ARID}$), as has been previously described (Gregory *et al.*, 1996), and the mutant ARID (GST- RETN/DRI$_{ARIDδH5}$) lacking the helix 5 sequence. Using elevated protein levels to detect any potential binding by the mutant, GST- RETN/DRI$_{ARID}$ was able to retard the available NP6 while GST-RETN/DRI$_{ARIDδH5}$ was not able to retard the NP6 DNA (Fig. 3A). We conclude that the RETN/DRI ARID is necessary for DNA binding *in vitro*.

The ARID Motif is Essential for RETN/DRI Function In Vivo

To test the *in vivo* importance of the ARID motif, the ARIDδH5 construct was placed downstream of a yeast GAL4 upstream activator sequence (UAS) and transformed into the *Drosophila* germline. Genetic crosses were used to place this construct under the transcriptional control of endogenous *retn/dri* enhancers via a *retn/dri*::GAL4 enhancer-trap P-element insertion line (Shandala *et al.,* 1999). When a wild-type *retn/dri* construct is expressed in this way, embryonic lethality of *retn/dri*^{1} homozygotes is rescued (Shandala *et al.,* 1999). However, when the ARIDδH5 construct was tested in the same assay, no rescue was observed. We conclude that the ARID domain is essential for RETN/DRI function *in vivo*.

RETN/DRI$_{ARIDδH5}$ Acts as a Dominant Negative

Failure of the *retn/dri* ARID deletion mutant to rescue the mutant phenotype demonstrated the *in vivo* requirement for the ARID domain. The RETN/DRI$_{ARIDδH5}$ construct used in this experiment had the capacity to interact with co-factors involved in RETN/DRI function. If so, the construct should act in an antimorphic, or dominant negative, way in the presence of the wild-type gene. To test for the antimorphic potential of this construct, wild-type RETN/DRI and RETN/DRI$_{ARIDδH5}$ lacking DNA-binding activity was expressed in the wild-type *retn/dri* pattern as described above, but now in the presence of the wild-type *retn/dri* gene. No flies were observed to carry both the UAS::*retn/dri*ARIDδH5 transgene and the *retn/dri::GAL4* driver, indicating that these genes are synthetically lethal, consistent with an antimorphic function.

In order to confirm the antimorphic activity of *retn/dri*ARIDδH5, we examined flies heterozygous for an amorphic *retn/dri* allele which were also expressing *retn/dri*ARIDδH5 in the wing, a non-essential *retn/dri*-expressing tissue. Examination of larval *retn/dri* expression

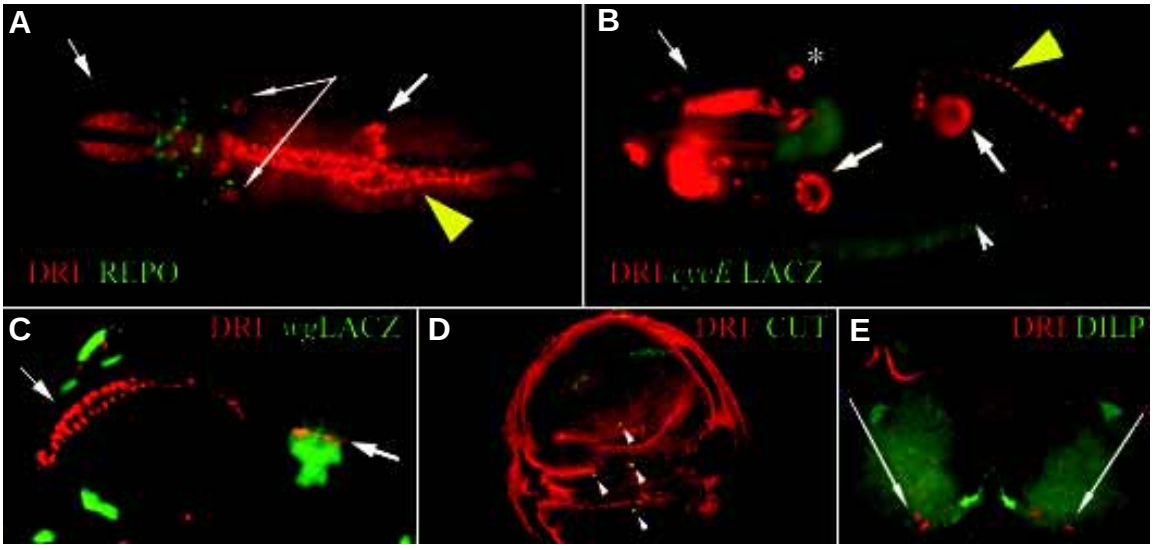

Fig 2. Developmentally regulated pattern of retn/dri expression in *Drosophila* embryos and larvae. *Immunochemical staining of whole mount embryos (A-C) or larval tissues (D,E) with rat-anti-DRI (red) (A-E). Images were captured using epifluorescence microscopy, except (D) which is a confocal image. Samples are counterstained with rabbit anti-REPO (green)* **(A)**, *rabbit anti-βgal (green)* **(B,C)**, *mouse anti-CUT (green)* **(D)** *and rabbit anti-DILP (green)* **(E)**. *Embryonic tissues that express* retn/dri *include the posterior region of the developing brain (A, long thin arrows), lateral glia and neurons of the ventral nerve cord (B, white arrowhead),* Drosophila *endocrine tissue of the ring gland (corpus allatum) (B, asterisk), pharyngeal muscles (A-C, short thin arrows), rings of the cells at major junctions along the gut (A-C, short thick arrows) and specific rows of cells that form part of the hindgut epithelium (A,B, yellow arrowheads). Expression in most of these tissues continues into larval development (e.g. cells in the brain in E, long thin arrows). Most of these cells are of unknown lineages and function. For instance, we failed to colocolise brain* retn/dri *positive cells with available markers such as the glial specific homeodomain protein REPO (A) and* Drosophila *Insulin-Like Peptide (DILP) (E). (D) Anti-Cut (green) and anti-RETN/DRI (red) staining in the larval wing imaginal disk. White arrowheads indicate the location of the sense organ precursor cells expressing both Cut and RETN/DRI.*

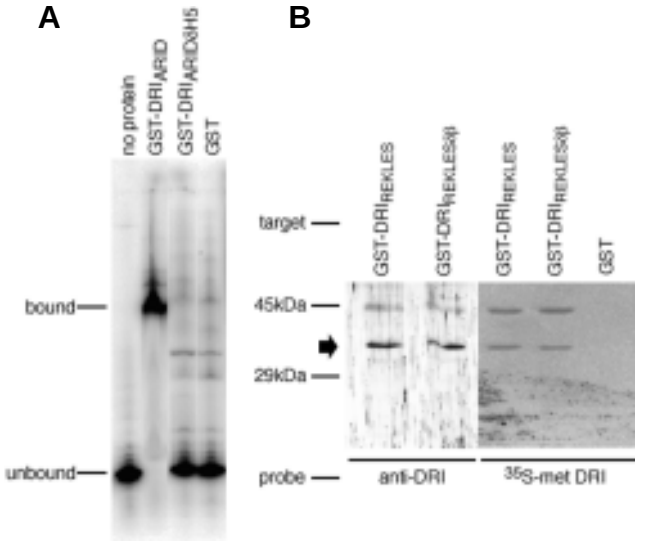

Fig. 3. *In vitro* analysis of eARID deletions. (A) *Gel electrophoretic mobility shift assay using GST, GST-RETN/DRI*$_{ARID}$ *and GST-RETN/DRI*$_{ARIDδH5}$ *proteins incubated with labelled NP6 oligonucleotide.* **(B)** *Protein blot assay for RETN/DRI REKLES domain self-association. GST-RETN/DRI*$_{REKLES}$ *and GST-RETN/DRI*$_{REKLESδβ}$ *were electrophoresed and transferred to nitrocellulose membranes. Membranes were probed with either anti-DRI antibody or radiolabelled full-length RETN/DRI protein, produced by* in vitro *transcription and translation. The 29kDa GST protein unfused to RETN/DRI and probed with radiolabelled RETN/DRI protein is also shown. Note that the GST-RETN/DRI*$_{REKLES}$ *and GST-RETN/DRI*$_{REKLESδβ}$ *proteins appear as two bands, both of which are less than the size predicted for a GST-RETN/DRI*$_{REKLES}$ *protein. The reason for this has not been established.*

patterns revealed that it is expressed in a subset of wing imaginal disk cells that express the homeodomain protein Cut (Fig. 2D). These *cut* and *retn/dri*-expressing cells are known to be the precursors of a group of sense organs, known as the campaniform sensilla, located on the wing (Huang *et al.*, 1991; Blochlinger *et al.*, 1993). Additionally, *retn/dri* appears to be expressed at low levels throughout the wing imaginal disk. Thus the wing was chosen as a target tissue for confirmation of *retn/dri*ARIDδH5 antimorphism.

UAS:: *retn/dri*ARIDδH5 expression was induced in developing wing cells using the *GAL471B* enhancer trap, which expresses *GAL4* in the wing-blade anlagen of the wing imaginal disk (Brand and Perrimon 1993). Expression of UAS::*retn/dri*ARIDδH5 under *GAL471B* control resulted in variable wing vein defects and losses of campaniform sensilla (Fig. 4 A-C), in regions consistent with the endogenous expression pattern of RETN/DRI (see Fig. 2D).

To establish whether *retn/dri*ARIDδH5 acts antimorphically, the dose of endogenous retn/*dri* was halved by placing *GAL471B* and *P*[UAS::*retn/dri*ARIDδH5] in a *retn/dri* heterozygous mutant background. Enhancement of the severity of the phenotype in *retn/dri*^{1} heterozygotes was observed (Fig. 4, compare D with E), confirming that the *retn/dri*ARIDδH5 construct was acting as an antimorphic form of the gene.

Other Effector Domains in eARID-Containing Proteins: the REKLES Domain

The eARID family proteins contain two additional conserved motifs, the REKLESα and REKLESβ domains (Fig. 1B, Kortschak *et al.*, 2000). The REKLES motif is located immediately carboxy-terminal to the eARID. It has a size of a little over 100 amino acids in the *C. elegans* and vertebrate RETN/DRI homologs, but in *Drosophila* there is a non-conserved insertion of 237 amino acids within the domain, dividing it into the α and β subdomains. A role for the REKLES domain in eARID protein function has been suggested by the observation that C-terminal deletions of the Bright protein, removing regions that include the REKLES domain, fail to tetramerise in gel retardation assays (Herrscher *et al.*, 1995).

The REKLESβ Region is not Required for Self-Association

To test whether the most conserved region of the REKLESβ domain was responsible for the homomerisation of RETN/DRI, the most highly conserved region of the RETN/DRI REKLES domain (amino acids 792 to 807), which includes the invariant residues of the REKLESβ region, was deleted by *in vitro* site-directed mutagenesis. A western blot of wild-type REKLES (GST–RETN/DRI$_{REKLES}$) and mutant REKLES (GST–RETN/DRI$_{REKLESδβ}$), was probed with *in vitro*

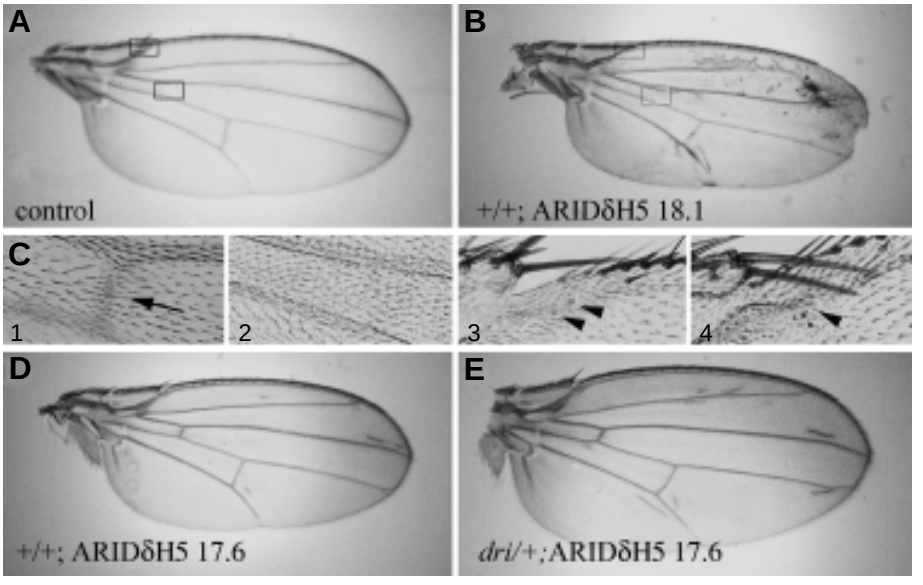

Fig. 4. Effects of mutant and wild-type retn/dri expression under GAL471B control in the wing-blade anlagen of the wing imaginal disk. *(A-F) Photomicrographs of wings from females of the following genotypes:* **(A)** w1118 *(full wing).* **(B)** *P[UAS::*retn/dri*ARIDδH5*18.1*]/*w1118*;+;*GAL471B*/+ (full wing),* **(C1)** *and* **(C2)** *higher magnifications of (A) and (B) respectively (bottom boxed area), showing the region around the anterior cross vein (black arrowhead in C1),* **(C3)** *and* **(C4)** *higher magnifications of (A) and (B) respectively (top boxed area), showing the region around the twin campaniform sensilla of the margin (black arrow heads indicate the presence of campaniform sensilla),* **(D)** w1118*; +;* GAL471B *P[UAS::*retn/dri*ARIDδH5* 17.6*]/+ and (E)* w1118*;* retn/dri1*/+;* GAL471B *P[UAS::* retn/dri*ARIDδH5* 17.6*]/+, showing an enhanced phenotype when the* retn/dri *gene dosage was halved.*

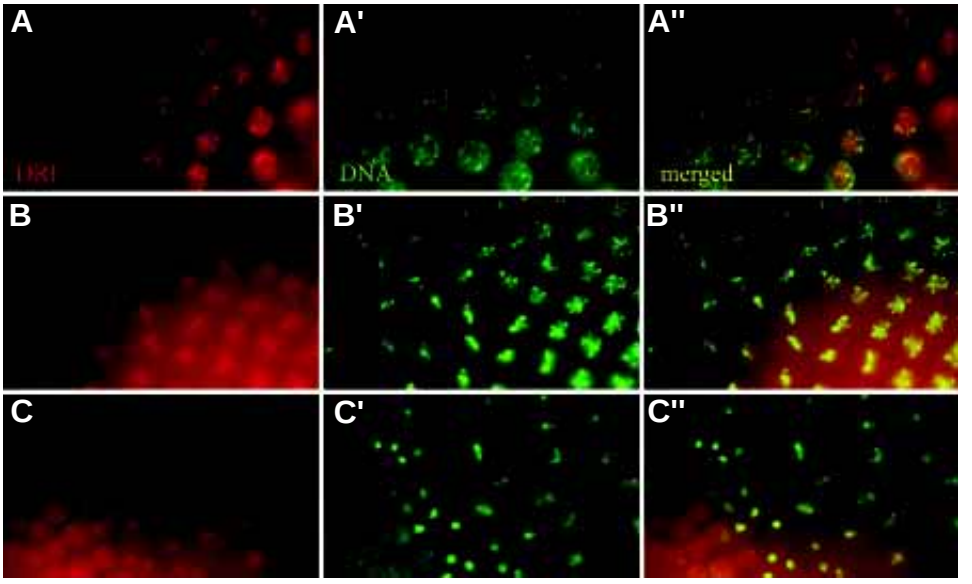

Fig. 5. RETN/DRI distribution during mitosis *RETN/DRI distribution, detected using polyclonal rat anti-RETN/DRI (red, A-C) during a wave of mitoses occurring in a syncytial embryo.* **(A)** *Speckles of RETN/DRI are observed in interphase nuclei.* **(B)** *At prophase/metaphase RETN/DRI can be visualised both on the chromosomes and diffusely at a low level in cytoplasm.* **(C)** *During anaphase, RETN/DRI appears to have degraded, before a high level of protein re-accumulates in the telophase nuclei. (A'-C'). The same sections stained forDNA with Hoechst 33258. (A''-C'') merged images.*

transcription/translated, ^{35}S-methionine-labelled RETN/DRI (Fig. 3B, arrow). No difference in signal intensity was observed between the wild-type and mutant forms, indicating that the REKLESβ region is not necessary for self-association.

retn/dri *Lacking the REKLESβ Region is able to Rescue* retn/dri *Function* In Vivo

In order to examine the *in vivo* role of the REKLESβ region of RETN/DRI, rescue experiments similar to those described for full length *retn/dri* and the ARID mutant form of *retn/dri* were performed. Unlike the ARID motif mutation, constructs lacking the REKLESβ region could still rescue the mutant phenotype. This shows that although it represents the most conserved part of the REKLES motif, the REKLESβ region of RETN/DRI is not essential for function during stages of embryonic and imaginal development rescued by the transgene. However, *in vivo* expression of retn/dri::GAL4>UAS::retn/driREKLESδβ in a heterozygous background caused a small but significant reduction in viability (data not shown), suggesting that this construct may have acted as a mild dominant negative form of the protein.

ARID Family Genes and Cell Cycle Regulation: A Role for retn/dri *in* Drosophila *Preblastoderm Mitoses*

retn/dri maternal products are uniformly distributed in the early *Drosophila* embryo during the preblastoderm cleavage stage, where nuclei are undergoing mitoses in a syncytium (Fig. 5, Gregory *et al.*, 1996). At interphase, the RETN/DRI protein is present in discrete apically located nuclear foci not completely overlapping with the highly condensed DNA foci stained preferentially by Hoechst 33258 dye (Fig. 5A). At metaphase, RETN/DRI can be visualised both on the chromosomes and at a low level in the cytoplasm (Fig. 5B). The cytoplasmic RETN/DRI gradually disappears during the anaphase/telophase transition, after which high levels of protein accumulate in the newly re-formed nuclei (Fig. 5C). After this stage, *retn/dri* is expressed in a range of terminally differentiating cells that have ceased proliferation (Fig. 2). Analysis of the zygotic *retn/dri* phenotype showed that *retn/dri* plays roles in many and perhaps all of the differentiating cells in which it is expressed (Shandala *et al.*, 1999; Shandala, Sibbons and Saint, unpublished observations).

To determine a function for *retn/dri* during the syncytial divisions, it is necessary to eliminate the maternal *retn/dri* product from the egg. This was achieved using mitotic recombination to produce *retn/dri* mutant clones of cells in the developing oocytes, using the method of the FLP-FRT-*ovo*^{D1} system (Chou and Perrimon, 1996). *retn/dri* mutant alleles were recombined onto chromosomes carrying FRT recombination sites at the base of chromosome arm 2R and placed *in trans* to a chromosome carrying a dominant female sterile mutation, *ovo*^{D1}, that prevents the production of eggs. A transgene expressing the FLP recombinase under the control of the heat shock promoter was used to induce recombination between non-sister chromatids of the two homologs. This resulted in the generation of *retn/dri* mutant cells that no longer carried the dominant female sterile allele *ovo*^{D1} and could therefore produce eggs.

Many early embryos derived from germline *retn/dri*$^{-}$ clones, and therefore lacking maternally-derived RETN/DRI, exhibited proliferation defects. The most pronounced of these defects was the loss of synchrony of cell divisions across the early syncytial embryo (Fig. 6 A-F, 7A-E). Affected embryos exhibited numerous anaphase bridges (Fig. 7 C,D) leading to the formation of aberrant nuclei, including micronuclei and the fusion of nuclei (Fig. 7 C-E). Cell cycle defects of this nature lead to a loss of cells during the syncytial divisions (Sullivan *et al.*, 1993), so it is not surprising that some embryos had fewer cells while still undergoing mitosis (Fig. 6 D-F, 7B,D). However, as previously observed for the zygotic *retn/dri* phenotypes, the germline clone embryonic proliferation defects were highly variable, with approximately 65% of blastoderm stage embryos exhibiting a normal distribution of nuclei during embryogenesis.

Discussion

The ARID family of genes are involved in a wide variety of transcriptional regulatory mechanisms and in a diversity of biological processes. The *retn/dri* gene of *Drosophila melanogaster* is perhaps the best characterised ARID family gene with respect to its role in development. In this report we extend this characterisation by describing studies of the *in vitro* and *in vivo* functions of conserved domains in the RETN/DRI protein. Gel mobility shift assays using the Engrailed consensus binding NP6 DNA sequence revealed that disruption of the ARID motif by deletion of the fifth α-helix disrupts DNA-binding. Similarly, dele-

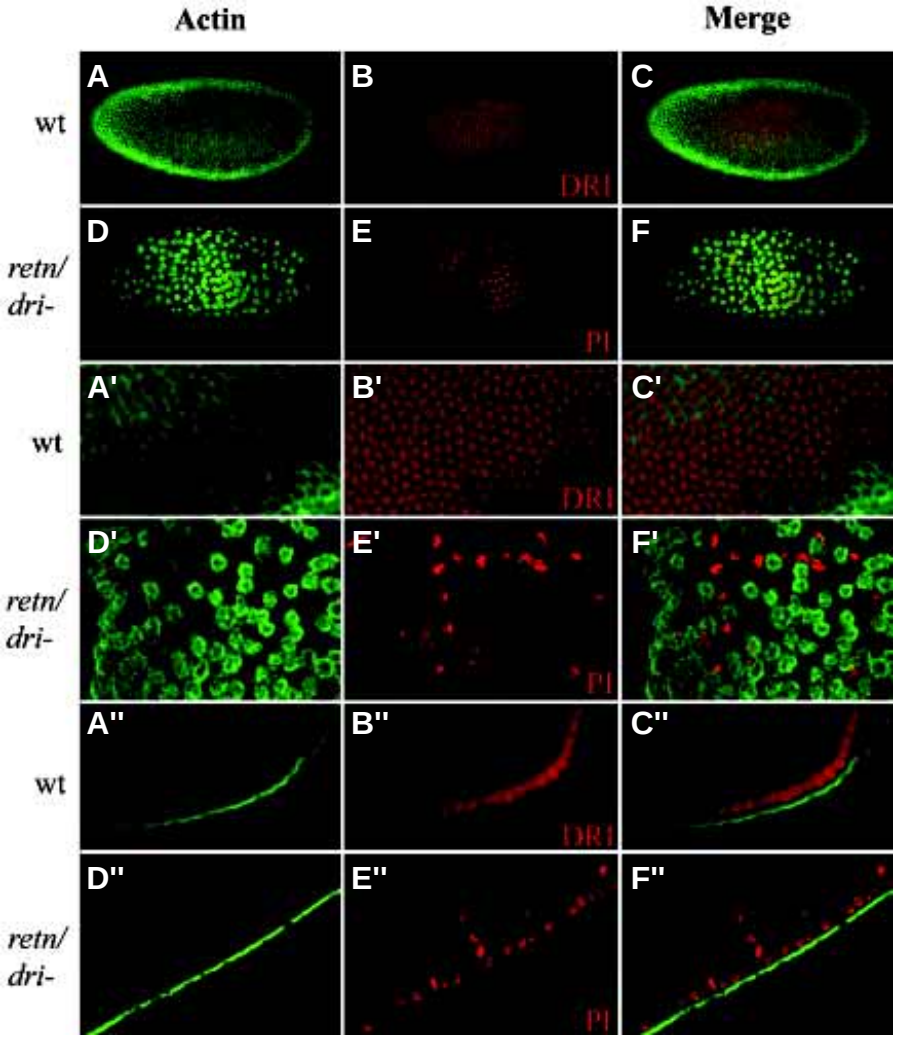

Fig. 6. Loss of nuclei in embryos lacking maternal RETN/DRI protein (A-C) *Wild-type embryos. Nuclei were labeled with anti-RETN/DRI (red), actin was detected with monoclonal mouse anti-Actin antibody (green).* **(D-F)** retn/dri *mutants. Nuclei were labeled with propidium iodine (PI, propidium iodide, red), anti-Actin staining is green.* **(A′- C′)** *Apical veiw of the wild-type embryos in (A-C).* **(D′-F′)** *Apical view of the* retn/dri *mutant embryos in (D-F), showing less cells and disruption of the normal relationship between F-actin and nuclear position in the syncytium.* **(A′′-C′′)** *Transverse optical section through the same wild-type embryo shown in (A-C).* **(D′′-F′′)** *Transverse optical section through the mutant embryo shown in (D-F), showing nuclei sinking into the interior of the embryo.*

tions of human SMARCF1 ARID and of helix 1 of mouse Bright dramatically abrogate DNA-binding activity of the resultant peptides (Herrscher *et al.,* 1995; Webb, 2001; Dallas *et al.*,2000). An *in vivo* requirement for ARID domain function was demonstrated by showing that the deletion of this same helix eliminated the ability of a *retn/dri* transgene to rescue the mutant phenotype. The *in vivo* requirement for ARID sequences has also been demonstrated for the mouse Desrt protein. Targeted deletion of the ARID-containing exon of Desrt in mice results in multiple developmental defects, including reduced embryonic viability, growth retardation, disruption of spermatogenesis and transient immune abnormalities (Lahoud *et al.*, 2001).

Examination of the phenotype of the deletion transgene in the presence of wild-type *retn/dri* in the present study showed that RETN/DRI$_{ARID\delta H5}$ acted as a dominant negative. This suggests that RETN/DRI acts as part of a protein complex, consistent with the observation that Bright tetramerises (Herrscher *et al.,* 1995) and that RETN/DRI forms protein complexes with the co-repressor Groucho (Valentine *et al.*, 1998).

eARID family members exhibit an independent, less well conserved bipartite domain termed the REKLES domain (Kortschak *et al.*, 2000). Western analysis of the REKLES domain using radio-labelled RETN/DRI as a probe showed that the REKLES domain is capable of mediating self-association, consistent with the finding of Herrscher *et al.* (1995) that the region of Bright corresponding to the REKLES domain confers tetramerisation activity. A requirement for the REKLESβ domain for RETN/DRI function *in vivo* was assayed by expression of RETN/DRI lacking the most conserved part of this subdomain. This construct rescued the *retn/dri* mutant phenotype as effectively as the full length *retn/dri* construct. This indicates that the REKLESβ deletion does not result in a significant loss of zygotic *retn/dri* activity, leaving us yet to demonstrate a function for this domain.

In the last of the studies reported here, we used germline clones to examine the role of maternal *retn/dri* products during the parasynchronous syncytial cleavage divisions that follow fertilisation of the *Drosophila* embryo. Embryos deprived of their maternally-derived *retn/dri* products in this way exhibit variable but frequent mitotic aberrations, including the loss of synchrony, the presence of incomplete sister chromatid separation and a reduction in the number of nuclei. RETN/DRI is present in cleavage stage nuclei, but appears to decrease in level during mitosis to accumulate in the nucleus after nuclear envelope formation. It is possible, therefore, that the mitotic phenotypes observed reflect a role for RETN/DRI in chromosome condensation in cleavage embryos, rather than mitosis itself.

A number of ARID family genes are implicated in the regulation of the cell cycle, particularly through interactions with the Retinoblastoma tumour suppressor protein pRB. RBP1 and RBP2, BDP and PLU-1 are all ARID-containing proteins that bind to human retinoblastoma binding proteins (Fattaey *et al.*, 1993; Adnane *et al.*, 1995; Koonin *et al.*, 1995; Lu *et al.*, 1999; Numata *et al.*, 1999; Quadbeck-Seeger *et al.*, 2000; Chan and Hong, 2001). One target of pRB repression is E2F, a transcription factor essential for expression of many genes involved in DNA synthesis and cell cycle control (reviewed by Nevins, 1998). Here, RBP1 recruits deacetylase mSIN3 complex to the pocket domain of pRB bound to E2F (Lai *et al.*, 2001)

Another member of the *Rbp* subfamily is the mouse *jumonji* gene. Mutation of the *jumonji* locus via a gene trap strategy caused death soon after birth, with severe developmental defects

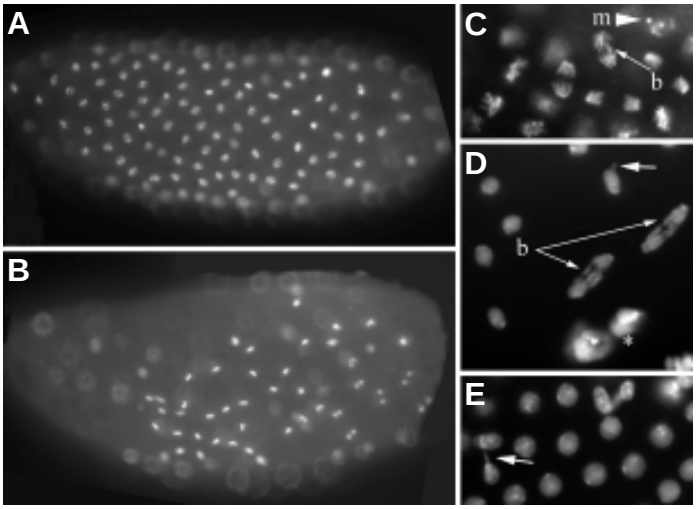

Fig. 7. Proliferation defects in embryos with no maternal retn/dri expression. *Whole mount embryos stained with Hoechst 33258.* **(A)** *Loss of synchrony of cell divisions across the early syncytial embryo.* **(B)** *Some embryos had fewer cells while still attempting mitosis.* **(C-E)** *Various mitotic defects observed in* retn/dri *mutant embryos: anaphase bridges (b, thin arrows), incomplete or aberrant nuclear reformation (short arrows) and formation of micronuclei (m, arrowhead).*

in the heart, liver, neural tube, spleen and thymus (Takeuchi *et al.*, 1995; Kitajima *et al.*, 2001). Detailed analysis of *jumonji* mutant tissues revealed that cardiac trabecular myocytes in the heart and lymphoid cells (megakaryocytes) in all hematopoetic tissues showed over-proliferation and overgrowth (Kitajima *et al.*, 2001).

retn/dri appears not to be expressed in all dividing cells, so it is unlikely that it is required in all mitotic divisions in the same way that RBP1 may be generally required. Furthermore, analysis of the phenotypes later in development has not revealed any requirement for RETN/DRI in later divisions, nor has it revealed any overgrowth phenotype that would indicate a negative regulatory role of the type inferred for mouse *jumonji*. However, the cleavage divisions in the *Drosophila* embryo are highly modified divisions, being syncytial and occurring every approximately 10 minutes. It is possible that RETN/DRI has been recruited to facilitate these extraordinarily rapid early embryonic mitotic cycles.

These studies confirm that the ARID motif is essential for *retn/dri in vivo* function, but the function of the more poorly conserved REKLES domain remains to be elucidated. The involvement of *retn/dri* in a variety of developmental processes, from the syncytial cleavage mitoses described here to the early axis patterning and later roles in muscle development (Shandala *et al.*, 1999) and neural development (T. Shandala, J. Sibbons and R.Saint, unpublished observation), may mean that the REKLES domain has a stage or role-specific function. Identification of other factors that bind RETN/ARID could provide a way of further exploring the function of the REKLES domain and of the RETN/DRI protein in general.

Materials and Methods

Generation of Domain-Specific Mutant retn/dri *Constructs*

To analyse the *in vitro* function of the ARID and REKLES domains, PCR amplification was used to generate pGEX::*retn/dri* constructs. Accuracy of PCR, and orientation and frame of insertion of the fragment were confirmed by sequence analysis. A deletion construct, pGEX::*retn/dri*ARIDδH5, lacking the putative eARID helix 5 was generated from pGEX::*retn/dri*ARID using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, California, USA). Mutant clones were confirmed by sequence analysis. Similarly, wild-type and mutant REKLES constructs were generated; fragments generated by PCR amplification were cloned into pGEX1 to generate pGEX::*retn/dri*REKLES (amino acids 400-901) and pGEX::*retn/dri*REKLESδβ (as above, but lacking the REKLESβ region) respectively. Orientation and frame of insertion of the fragments were confirmed by sequence analysis.

For analysis of the *in vivo* function, a full length cDNA *retn/dri* clone (Gregory *et al.*, 1996) was mutagenised using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, California, USA) to generate the *retn/dri* mutant plasmids p*retn/dri*ARIDδH5 and p*retn/dri*REKLESδβ respectively. Mutated sites were confirmed by sequence analysis. Mutant *retn/dri* cDNAs were cloned into p*P*[UAST] for *Drosophila* germline transformation (Spradling and Rubin, 1982) and GAL4-induced expression (Brand and Perrimon, 1993). Correct orientation of the cDNA was confirmed by restriction analysis. *In vivo* GAL4-induced expression of wild-type and mutant forms of RETN/DRI was confirmed by western analysis of eye imaginal disks expressing RETN/DRI under the control of the *GMR::GAL4* transgene.

Generation of Germline retn/dri *Mutant Clones*

To abolish the maternal *retn/dri* contribution, embryos derived from *retn/dri*^{1} and *retn/dri*^{2} germline clones were generated using the FLP-FRT-*ovo*^{D1} system developed by Chou and Perrimon (1996).

Immunochistochemistry

The following antibodies were used in these studies: polyclonal rat anti-DRI (Gregory *et al.,* 1996), polyclonal anti rabbit-βgal (Rockland Immunochemicals, California, USA), monoclonal anti-CUT, anti-actin (Developmental Studies Hybridoma Bank, University of Iowa, USA), rabbit anti-DILP (a gift from Mark R.Brown, Department of Entomology, University of Georgia, Athens, USA) and anti-REPO (a gift from Andrew Travers, Laboratory of Molecular Biology, Medical Research Council, Cambridge, UK).

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