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Dscam diversity is essential for neuronal wiring and self-recognition

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Abstract

Neurons are thought to use diverse families of cell-surface molecules for cell recognition during circuit assembly. In *Drosophila*, alternative splicing of the *Down syndrome cell adhesion molecule* (*Dscam*) gene potentially generates 38,016 closely related trans-membrane proteins of the immunoglobulin superfamily, each comprising one of 19,008 alternative ectodomains linked to one of two alternative transmembrane segments¹. These ectodomains show isoform-specific homophilic binding, leading to speculation that Dscam proteins mediate cell recognition². Genetic studies have established that Dscam is required for neural circuit assembly^{1,3-10}, but the extent to which isoform diversity contributes to this process is not known. Here we provide conclusive evidence that Dscam diversity is essential for circuit assembly. Using homologous recombination, we reduced the entire repertoire of Dscam ectodomains to just a single isoform. Neural circuits in these mutants are severely disorganized. Furthermore, we show that it is crucial for neighbouring neurons to express distinct isoforms, but that the specific identity of the isoforms expressed in an individual neuron is unimportant. We conclude that Dscam diversity provides each neuron with a unique identity by which it can distinguish its own processes from those of other neurons, and that this self-recognition is essential for wiring the *Drosophila* brain.

The complexity and specificity of neuronal wiring implies the existence of a cellular recognition code that allows neurons to distinguish between one another¹¹. It has been speculated that families of highly diverse cell-surface molecules could provide this function, such as the vertebrate neurexins¹², cadherins¹³ and cadherin-related neuronal receptors¹⁴, and the insect Dscams^{1,15}. However, it remains unclear to what extent the molecular diversity of such proteins is essential for wiring specificity, and how their diversity contributes to neuronal recognition. In *Drosophila melanogaster*, as many as 38,016 Dscam isoforms are generated by alternative splicing¹. Each isoform consists of an ectodomain containing a unique combination of three different variable immunoglobulin-like domains

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Author Contributions B.J.D. designed the targeting strategy, and E.D., E.V. and B.J.D. generated the Dscam^{single} alleles, which were verified by E.D., D.H. and H.W.K. All biochemical and phenotypic analyses were performed by D.H., together with H.W.K. The intragenic MARCM strategy was conceived by D.H., E.D., H.W.K. and S.L.Z. B.J.D., D.H. and S.L.Z. wrote the manuscript.

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linked to one of two alternative transmembrane segments (Fig. 1a). The variable ectodomain segments are encoded by 12, 48 and 33 alternatives for exons 4, 6 and 9, respectively, whereas the transmembrane domain is encoded by two versions of alternative exon 17 (Fig. 1a). A given ectodomain isoform binds strongly to itself, but only weakly, if at all, to other isoforms². Thus, *Dscam* diversity could provide a molecular mechanism for selective recognition among neurons.

The potential role of *Dscam* diversity in neuronal wiring has previously been approached using various deletion mutations that remove subsets of alternative versions of exon 4 from the genomic locus. These alleles reduce the potential ectodomain diversity at most from 19,008 to some 5,000 isoforms. All of these mutants develop to adulthood and are fertile, and their nervous system organization is largely normal^{8,16}. In one study, reducing the potential ectodomain diversity to ~11,000 isoforms resulted in an increase in the variability of axon branching and the appearance of some ectopic branches in an identified somatosensory neuron⁵, alluding to a specific role for subsets of isoforms. However, because different cells splice *Dscam* differently^{7,17}, these genomic deletions may result in variable reduction of *Dscam* protein levels within different cells. For example, a specific isoform of N-cadherin is required for targeting of R7 neurons in the visual system, but this is due to cell-specific splicing rather than an isoform-specific function¹⁸. It is therefore unclear whether the defects observed in *Dscam* isoform deletion mutants reflect a functional requirement for specific isoforms, a mosaic *Dscam* loss-of-function, or both. Thus, whether *Dscam* diversity is essential for neural circuit assembly remains a crucial and unresolved issue. A definitive test to address the importance of ectodomain diversity would be to completely eliminate alternative splicing, reducing *Dscam* ectodomain diversity to just a single isoform expressed from the endogenous locus.

We used homologous recombination to replace the genomic region encoding the variable ectodomains with a complementary DNA encoding a single isoform (Fig. 1a and Supplementary Fig. 1). Three distinct ectodomains were arbitrarily selected. These were shown to exhibit homophilic binding (Fig. 1b), a property shared with all other isoforms we have studied (>100) (refs 2; 19). We refer to these mutant alleles collectively as *Dscam*^{single}, as the phenotypes of all three alleles were similar, and individually by indicating the selected exon variants in the allele name (for example, *Dscam*^{3.31.8} contains the variable exons 4.3, 6.31, and 9.8). All of these alleles carry an FRT (FLP recombinase target) sequence inserted in the intron between exons 16 and 17. Accordingly, we also generated a control allele, *Dscam*^{FRT}, which has an FRT insertion in the same location but retains the full complement of alternative exons (Fig. 1a). We verified the intended genomic rearrangements by sequencing 14 kb from the *Dscam* locus in each of these alleles. Sequencing of cDNAs confirmed that each *Dscam*^{single} allele only expresses the designated ectodomain isoform, whereas *Dscam*^{FRT} expresses many different isoforms. Protein levels (Fig. 1c) and localization (Fig. 1d), as well as the relative use of the two alternative transmembrane exons (Fig. 1e) were similar among the *Dscam*^{single} alleles and the *Dscam*^{FRT} and wild-type controls.

Like *Dscam*^{null} alleles¹, all three *Dscam*^{single} alleles are recessive lethal. Thus, not only is *Dscam* itself essential, but so is its diversity. To assess more carefully the viability of these mutants, newly hatched mutant larvae were isolated and grown without potential competition from their siblings. Under these conditions, 17–22% of *Dscam*^{null} homozygous animals survived until late pupal stages (Fig. 2a). In contrast, as many as 76–89% of *Dscam*^{single}/*Dscam*^{null} animals survived to the pupal stages, suggesting that at least some *Dscam* function is diversity-independent. Notably, *Dscam*^{single} homozygotes died in early larval development, regardless of the isoform encoded (Fig. 2a). Axonal pathways were disrupted in the embryonic ventral nerve cord in all of these mutant combinations, and this

phenotype too was most severe in the *Dscam*^{single} homozygotes (Fig. 2b). The phenotypes in animals carrying two different *Dscam*^{single} alleles (that is, transheterozygous animals) have a similar spectrum of phenotypes to *Dscam*^{single} homozygotes (Fig. 2a, b).

To explore further the neuronal wiring in these mutants, we examined the synaptic organization of the olfactory system (Fig. 2c-e). Olfactory receptor neurons (ORNs) project axons into the antennal lobe, where they form synapses with the dendrites of projection neurons and local interneurons. Typically, neurons in a given ORN class all express the same odorant receptor, and form connections within a distinct synaptic module, called a glomerulus²⁰. *Dscam* is required autonomously in ORNs, projection neurons and interneurons to establish this circuitry^{4,6}. In *Dscam*^{null} homozygotes, we observed a highly characteristic phenotype in which axons of a given ORN class target more than one glomerulus in each antennal lobe (Fig. 2d). Discrete glomeruli still formed in these mutants, but their stereotyped arrangement within the lobe was highly disrupted. In marked contrast, the antennal lobe of all *Dscam*^{single}/*Dscam*^{null} animals was highly disorganized with no distinct glomerular structure, and ORNs formed many ectopic termini throughout the antennal lobe (Fig. 2e). Taken together, these data establish that *Dscam* diversity is essential for neural circuit formation.

What is the role of *Dscam* diversity in circuit assembly? One possibility is that diversity is instructive, with distinct isoforms specifying distinct synaptic connections¹. This might be an attractive model, but there is little evidence to support it. An alternative model²¹ posits that *Dscam* diversity might provide a molecular mechanism for 'self-avoidance', that is, the propensity of multiple dendritic or axonal branches from the same neuron (sister branches) to avoid each other and thereby elaborate appropriate receptive or terminal fields, respectively^{22,23}. This phenomenon requires recognition and repulsion between self-neurites, but not between neurites of different neurons. *Dscam* diversity might provide this function, because each neuron expresses a unique set of *Dscam* isoforms^{7,17}, and isoform-specific homophilic binding mediates repulsion^{2,8}. To test this model, we focused on the simple and well-characterized sister branch segregation of mushroom body neurons in the central brain.

Each mushroom body comprises thousands of neurons. During development, each of these neurons extends an axon in a fascicle called the peduncle, at the end of which it bifurcates to produce two sister branches, one segregating into the dorsal lobe and the other into the medial lobe (Fig. 3a). Because many axons bifurcate at the same time and in close proximity to each other, each branch must be able to distinguish its sister branch from branches of other neurons, to ensure a high fidelity of sister branch segregation. *Dscam* is essential for this process³, and each mushroom body neuron expresses a distinct array of *Dscam* isoforms⁷. To satisfy the model that *Dscam* diversity promotes self-recognition and self-avoidance of sister branches²¹, two critical conditions must be met. First, the specific set of isoforms expressed in a given mushroom body neuron should not be important. Second, and in contrast, whatever set of isoforms a given mushroom body neuron expresses, it should be essential that this set is different from that of its neighbours. Transgenic rescue experiments and deletion mutant analysis have confirmed the first condition^{7,16}. However, this condition on its own is also consistent with the null hypothesis that *Dscam* diversity is not required at all in mushroom body neurons. Thus, the critical test of the self-recognition model is to establish whether sister branch segregation requires neighbouring mushroom body axons to express different sets of *Dscam* isoforms.

To test this prediction, we examined mushroom body morphology in *Dscam*^{single} and control animals (Fig. 3b-e). In *Dscam*^{FRT} controls, the normal bi-lobed structure of the mushroom body was observed, with two lobes representing populations of sister branches

that had segregated correctly (Fig. 3b). In sharp contrast, this normal bi-lobed mushroom body morphology was never observed in *Dscam*^{single} animals. In some 97% of *Dscam*^{single}/*Dscam*^{null} mushroom bodies analysed, one of the two lobes was completely absent—typically the dorsal lobe (Fig. 3d). In the few remaining samples, one mushroom body lobe was significantly thinner than the other. We also observed a strong, but less severe, phenotype in ~90% of *Dscam*^{single}/*Dscam*⁺ heterozygotes (Fig. 3c), with one mushroom body lobe either absent (~60%) or thinner (~30%), suggesting that some mushroom body sister branches segregate normally, but most do not. This dominant phenotype indicates that the mushroom body defects observed in *Dscam*^{single}/*Dscam*^{null} animals do not result from the loss of any one isoform, but rather the presence of the same isoform on all axons. Thus, *Dscam* diversity is essential for mushroom body sister branch segregation, which is compromised even if only ~50% of *Dscam* proteins in each neuron represent a single isoform shared by all neurons.

Although these data support a role for *Dscam* diversity in mushroom body branch segregation, it remains possible that the isoforms encoded in these *Dscam*^{single} alleles are simply non-functional or even inhibitory for this process. To address this issue, we generated mosaic animals in which a single mushroom body neuron expresses only one *Dscam* isoform from a *Dscam*^{single} allele, but all other neurons express the full complement of isoforms from the *Dscam*^{FRT} allele. This was achieved using an intragenic variation of the MARCM technique²⁴, in which mitotic recombination was induced between FRT sites within two modified *Dscam* alleles (Fig. 4a and Supplementary Fig. 1). We tested two different *Dscam*^{single} isoforms with this intragenic MARCM system, and found that the sister branches of isolated *Dscam*^{single} mushroom body neurons segregated with high fidelity (Fig. 4b), but that sister branches of *Dscam*^{null} neurons did not³ (Fig. 4b). This experiment establishes that the single ectodomains we selected do indeed support sister branch segregation. Therefore, the lack of segregation in animals with a *Dscam*^{single} allele must be due to the loss of diversity, not the loss of *Dscam* function. Taken together, these results demonstrate that *Dscam* diversity is dispensable within a single neuron, but essential within a population of neurons, supporting the notion that it provides each neuron with a unique cell-surface identity.

In conclusion, here we provide strong evidence that *Dscam* diversity is critical for neuronal wiring. We envision that *Dscam* diversity contributes to wiring specificity in many different ways. One of these is self-recognition and self-avoidance, as demonstrated here for mushroom body neurons. We propose that this is a central function for *Dscam* diversity in neural circuit formation.

METHODS SUMMARY

The strategy used to generate the *Dscam*^{single} and *Dscam*^{FRT} alleles is indicated in Supplementary Fig. 1, and is based on the ends-in targeting strategy detailed in ref. 25. Three isoforms were selected essentially at random from sequenced cDNAs, with the only criterion being to select three distinct variants for each of exons 4, 6, and 9. Biochemical and molecular characterization of these alleles were performed as described previously^{2,7,8}. For survival analysis, each *Dscam* mutant animal was genotyped at first to second instar larval stages using *kruppel*-GFP, *CyO* balancer, and then transferred to fresh grape plates with a thin spread of yeast paste and raised at room temperature (~22–25 °C). Independently isolated alleles harbouring the same isoform were used to generate homozygous *Dscam*^{single} animals to avoid effects from potential second site mutations. Embryonic ventral nerve cords were immunostained as described previously⁷. For other analyses, animals that survived until late pupal stage were used. Immunostainings of pupal or adult brains were performed as described previously^{6,7}. For intragenic MARCM analysis, clones were generated by

inducing heat-shock-mediated expression of FLP recombinase at late larval to early pupal stages. Heat-shock was carried out at 37 °C for 1 h. *Dscam*^{null} mutant clones were generated using conventional MARCM as previously described⁷.

METHODS

Generation of *Dscam*^{single} alleles

Homology regions were amplified by PCR from genomic DNA extracted from the reference strain for the *Drosophila* genome project²⁶, and cloned into custom-built targeting vectors. For the single-ectodomain constructs, RT-PCR was performed on total RNA extracted from the heads of Canton-S adults, amplifying exons 3–11 and inserting this fragment in-frame into the flanking genomic region within the targeting vector, using an endogenous Asp 718 site in exon 3 and an *NheI* site engineered into exon 11, without altering the predicted amino acid sequence. Donor insertions on the X or 3rd chromosome were obtained by P-element-mediated transformation. These donor elements were excised and linearized with *hsFLP* and *hsI-SceI*, respectively²⁵. The resulting virgin females were crossed to *eyFLP* males²⁷, and reintegration of the targeting element was detected in the progeny by the presence of an *eyFLP*-resistant *white*⁺ marker (that is, flanked by a single FRT, rather than the two FRTs of the donor element). The final alleles were then obtained by FLP-induced recombination, as indicated in Supplementary Fig. 1, selecting for loss of the *white*⁺ marker. Two independently derived isolates were established for *Dscam*^{3.31.8} and *Dscam*^{10.27.25} alleles, obtained in turn from two independently derived intermediate alleles. One allele was generated for *Dscam*^{6.5.9}.

Biochemical and molecular characterization

The bead aggregation assay was performed as previously described². For immunoblots, approximately five brains dissected from the third instar larvae were homogenized directly in SDS sample buffer and western blots were performed using rabbit anti-Dscam antibody¹ at 1:1,000 dilution. The membrane was re-probed with a 1:1,000 dilution of rabbit anti-actin antibody (Sigma). RT-PCR analysis to characterize use of alternative ectodomain isoforms was performed as previously described⁷. The use of exons 17.1 and 17.2, encoding alternative transmembrane domains, was characterized by RT-PCR with primers against exon 16 and exon 18, followed by gel electrophoresis separation of PCR products containing exon 17.1 or 17.2.

Immunohistology

The following antibodies were used for immunohistology: rabbit anti-Dscam (1:500; ref. 7), monoclonal antibody 1D4 (anti-FasII; 1:10), rabbit anti-GFP (1:1,000, Molecular Probes), monoclonal antibody nc82 (1:10; ref. 28), Cy5-conjugated goat anti-HRP (1:200, Jackson ImmunoResearch Laboratories) and Alexa488- or Alexa568-conjugated goat anti-mouse or anti-rabbit (1:200, Molecular Probes). Late pupal or adult brains were dissected, and immunostaining was performed essentially as previously described^{6,7}. Stage-16 embryos were fixed and immunostained as previously described⁷. Immunofluorescent samples were analysed using an LSM 510 Meta (Zeiss).

Fly stocks

Dscam-null alleles (*Dscam*²¹, *Dscam*²³ and *Dscam*^{Df6055}) have been described previously^{1,6}. For antennal lobe phenotypic analyses, transgenic lines were generated, each containing a putative promoter of an *Or* gene (for example, *Or47a*) fused to a synaptotagmin-GFP marker (for example, *Or47a-sytGFP*). Three *Or-sytGFP* transgenes

were used for this study (*Or47a-sytGFP*, *Or47b-sytGFP* and *Gr21a-sytGFP*) (X. L. Zhan, P. Cayirlioglu, I. C. Grunwald, D. Gunning and S.L.Z, unpublished reagents).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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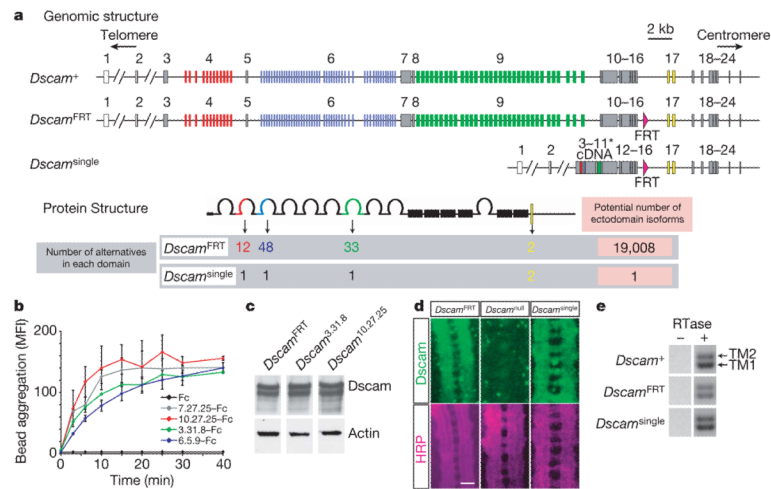
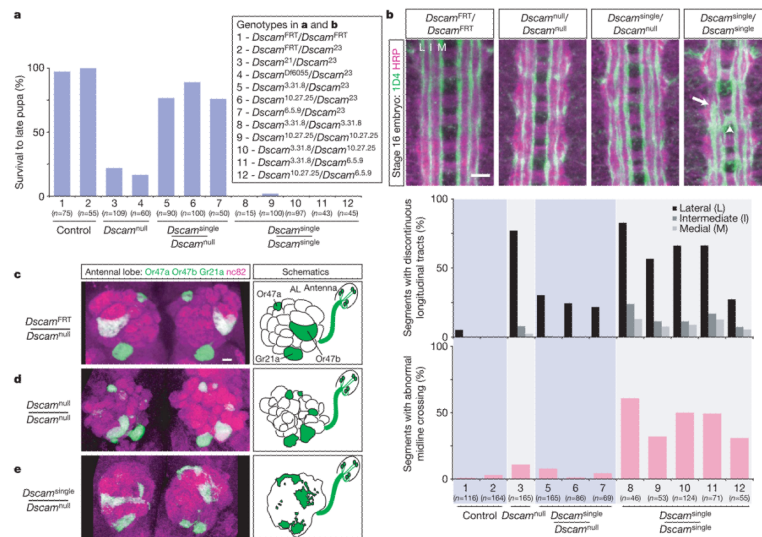


Figure 1. Generation and molecular characterization of *Dscam*^{single} alleles

a, Schematic representation of the genomic organization and proteins encoded by *Dscam* alleles used in this paper. Numbers above bars in the genomic structure indicate exons. Alternatively spliced exons are shown in colour. cDNA encoding a single isoform in the *Dscam*^{single} includes exons 3–11 (asterisk). Pink triangles indicate the FRT site between exons 16 and 17. **b**, Using a bead-aggregation assay, *Dscam*^{10.27.25}, *Dscam*^{3.31.8}, and *Dscam*^{6.5.9} ectodomains show homophilic binding similar to the *Dscam*^{7.27.25} control². Aggregation of fluorescent beads decorated with ectodomain–Fc fusion proteins was measured as an increase in the mean fluorescence intensity (MFI) of each particle. Binding experiments were performed twice. Error bars represent ± 1 s.d. **c**, *Dscam*^{single} alleles express Dscam protein at wild-type levels. The level of Dscam protein in extracts of the larval central nervous system was assessed by immunoblotting. Actin was used as a loading control. **d**, Dscam protein expression pattern (green) is normal in *Dscam*^{single} embryonic ventral nerve cord (stage 16). Anti-HRP (horseradish peroxidase) staining (purple) was used to visualize the neuropil. Scale bar, 10 μm. **e**, Expression of two alternative transmembrane (TM) domains in *Dscam*^{single} animals. RT-PCR across exons encoding each of the alternative TM domains in *Dscam*^{single} animals. RT-PCR across exons encoding each of the alternative TM domains was performed using RNA extracted from the third instar larval brains. Gel electrophoresis separates products encoding TM1 and TM2 as indicated.



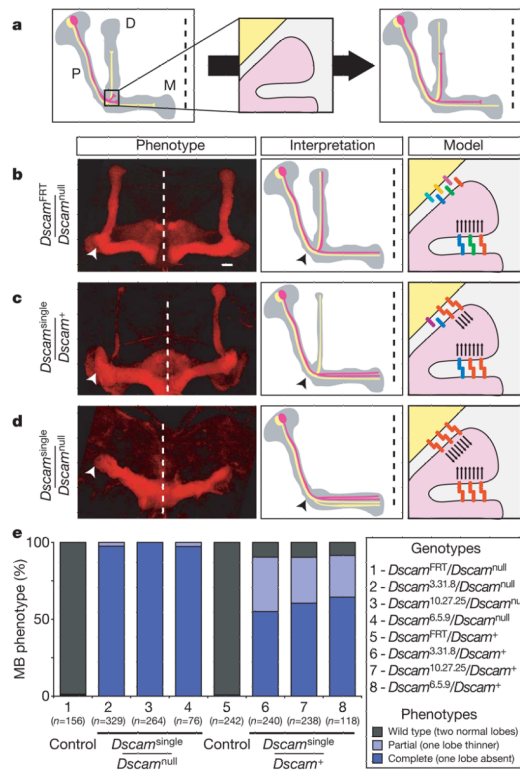


Figure 3. *Dscam* diversity is required for mushroom body development

a, Schematic of mushroom body development (P, peduncle; D, dorsal lobe; M, medial lobe). Two representative mushroom body neurons are shown (yellow and pink). **b–d**, Left panels show mushroom body lobes from late pupae visualized by staining with monoclonal antibody 1D4 (anti-FasII). Only the lobe region is shown. Arrowhead indicates the location of the branch point. Dashed line, midline. Scale bar, 10 μ m. *Dscam* isoforms expressed on sister branches (pink) and a non-self branch (yellow) are represented as coloured bars in panels on the right. Arrows indicate repulsive signals resulting from *Dscam* isoform-specific homophilic binding. **b**, Control animals (*Dscam^{FRT}/Dscam^{null}*). **c**, *Dscam^{single}/Dscam⁺* heterozygous animals. **d**, *Dscam^{single}/Dscam^{null}* mutant animals. **e**, Quantification of mushroom body (MB) phenotypes.

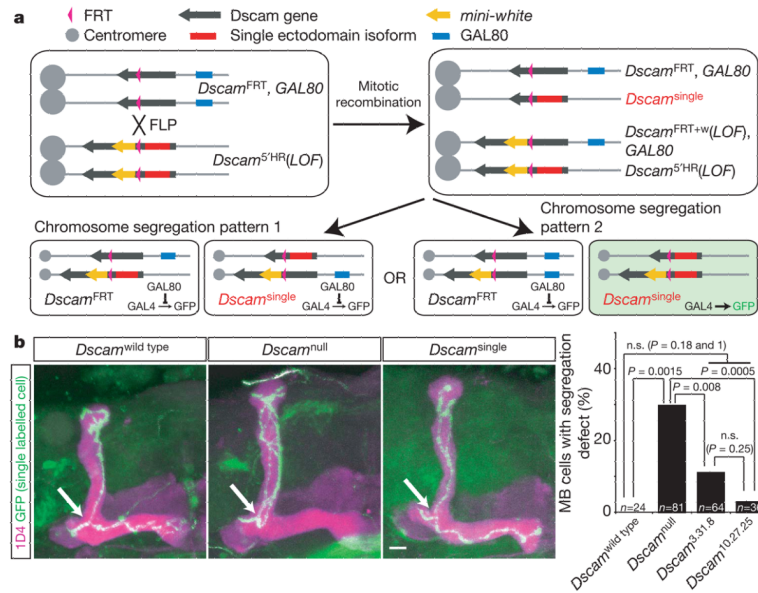


Figure 4. *Dscam*^{single} is sufficient to promote branch segregation with high fidelity at the single-cell level

a, Schematic of intragenic MARCM strategy to generate and label single *Dscam*^{single} cells in an otherwise wild-type background (that is, transheterozygous with the *Dscam*^{FRT} and *Dscam* loss-of-function (*Dscam*^{LOF}) alleles). FLP recombinase induces mitotic recombination between FRT sites within the *Dscam* locus. Chromosomes can segregate in two ways. In one way (pattern 2), GFP-labelled (green) *Dscam*^{single}/*Dscam*^{5'HR} mutant cells are generated. The *Dscam*^{5'HR} allele (Supplementary Fig. 1) is a loss-of-function allele. If chromosomes segregate in the alternative fashion (pattern 1), no labelled cells will be produced. One of these cells will carry an intact *Dscam*^{single} allele. **b**, Branch segregation phenotypes. Labelled cells for *Dscam*^{wild type} and *Dscam*^{single} were generated using intragenic MARCM, and *Dscam*^{null} labelled cells were produced using conventional MARCM. The mushroom body was visualized by staining with monoclonal antibody 1D4, anti-FasII (purple), and the clones were labelled with membrane-targeted chimeric GFP (mCD8GFP, green)²⁴. The genotype of each clone is indicated above the panel. Arrows indicate the branch point. Quantification is shown as a bar graph (using a two-tailed Fisher's exact test; n.s., not significant).