Evidence for a novel function of Awd in maintenance of genomic stability

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The abnormal wing discs (awd) gene encodes the Drosophila homolog of NME1/NME2 metastasis suppressor genes. Awd acts in multiple tissues where its function is critical in establishing and maintaining epithelial integrity. Here, we analysed awd gene function in Drosophila epithelial cells using transgene-mediated RNA interference and genetic mosaic analysis. We show that awd knockdown in larval wing disc epithelium leads to chromosomal instability (CIN) and induces apoptosis mediated by activation of c-Jun N-terminal kinase. Forced maintenance of Awd depleted cells, by expressing the cell death inhibitor p35, downregulates atypical protein kinase C and DE-Cadherin. Consistent with their loss of cell polarity and enhanced level of matrix metalloproteinase 1, cells delaminate from wing disc epithelium. Furthermore, the DNA content profile of these cells indicates that they are aneuploid. Overall, our data demonstrate a novel function for awd in maintenance of genomic stability. Our results are consistent with other studies reporting that NME1 down-regulation induces CIN in human cell lines and suggest that Drosophila model could be successfully used to study in vivo the impact of NME/Awd-induced genomic instability on tumour development and metastasis formation.

Genomic stability is critical for cell survival and development and several cellular mechanisms act to maintain genomic integrity1. Failure of these mechanisms underlies aging and can lead to malignancies such as cancer2 and age-related neurodegenerative diseases3. Chromosomal instability (CIN) is a form of genomic instability that often leads to aneuploidy, a deleterious condition characterised by copy number changes affecting part or whole chromosomes4. Several dysfunctions could lead to CIN5. Defective activity of the spindle assembly checkpoint (SAC), a signalling pathway that blocks anaphase onset in response to mis-attachment of chromosomes to the mitotic spindle, leads to CIN and aneuploidy6. Work in Drosophila showed that loss of function of SAC genes as well as loss of function of genes involved in spindle assembly, chromatin condensation and cytokinesis induce CIN7. More recent work in larval disc epithelia has shown that down-regulation of these genes causes apoptotic cell death through activation of the c-Jun N-terminal kinase (JNK) pathway8,9. Interestingly, blocking CIN-induced apoptotic cell death induces tumourigenic behaviour including basement membrane degradation, cell delamination, tissue overgrowth and aneuploidy.

The abnormal wing discs (awd) gene encodes the Drosophila homolog of NME1/2 metastasis suppressor genes. Awd is a well-known endocytic mediator whose function is required in multiple tissues during development10. Genetic studies showed that Awd endocytic function ensures appropriate internalisation of chemotactic signalling receptors such as platelet-derived growth factor/VEGF receptor (PVR)11 and fibroblast growth factor receptor (FGFR) and thus it regulates invasion and cellular motility12. Furthermore, this endocytic function regulates Notch receptor trafficking13 and is required for maintenance of epithelial integrity as it controls the turnover of adherens junction components in ovarian somatic follicle cells14. Consistent with the high degree of functional conservation between Awd and its mammalian counterparts, recent studies have shown a role for the NME1/2 proteins in vesicular transport15.

In the present work, we have extended our analysis of the functional conservation between Awd and NME1/2 proteins. Since loss of NME1 gene function in human cell culture leads to polyploidy16, we have explored the role of Awd in maintenance of genomic stability. Our data show that knockdown of awd in wing disc cells leads to CIN.
and to the CIN-induced biological responses mediated through JNK activation. Furthermore, when combined with block of apoptosis, down-regulation of awd leads to cell delamination and aneuploidy. Thus, the results of our in vivo analysis show a novel function of awd in maintenance of genomic stability.

**Results**

**Down-regulation of awd leads to genomic instability and cell death.** We have analysed the effects of Awd depletion in larval wing discs since these primordia are an excellent model system to study CIN and tumourigenesis. As shown in Fig. 1A, the Awd protein is expressed throughout the wing disc (n = 30). We have down-regulated awd expression through UAS/Gal4-driven RNA interference. The engrailed-Gal4 (en) driver has been used to induce expression of the UAS-awd-RNAi (awdi) construct in the posterior compartment cells of larval wing disc (Fig. 1A). The use of the compartment specific en-Gal4 driver allows direct comparison of wild type cells in the anterior compartment versus awd mutant cells in the posterior compartment within a single imaginal disc. Co-expression of the GFP marker allows easy recognition of the domain targeted for awdi silencing in en > GFP, awdi larvae (hereafter referred as en > awdi). To mark the anterior domain, we have stained wing discs for cubitus interruptus (ci), whose expression is repressed in the posterior domain by the en gene product (Fig. 1B). The analysis of en > awdi wing discs has shown patches of cells lacking GFP expression and expressing Awd and Ci protein despite their localisation in the posterior region of the disc where Awd is downregulated (73.3% wing discs; n = 30) (Fig. 1B). Thus, a genomic instability event has involved the second chromosome hosting the en driver leading to impaired en driver activity and loss of heterozygosity of the UAS-GFP and UAS-awdi transgenes. Furthermore, expression of the Ci anterior marker in these GFP+, Awd+ cells located in the posterior wing disc domain, demonstrates impaired en gene function. Thus, down-regulation of awd expression leads to genomic instability events that involve both copies of the second chromosome and cause loss of en gene function.

Further analyses of en > awdi wing discs have shown that down-regulation of awd in posterior domain activates JNK as indicated by the presence of the phosphorylated form of JNK (pJNK) (Fig. 1C, n = 35) and by the elevated level of matrix metalloproteinase 1 (MMP1) (Fig. 1D, n = 39), a downstream effector of JNK pathway. Furthermore, these posterior cells exhibit an increased level of apoptotic death as shown by cleaved-Caspase 3 (Casp3) staining (Fig. 1E, n = 30). Genomic instability often leads to CIN and aneuploidy. Interestingly, CIN induced by silencing of SAC genes, or downregulation of genes involved in spindle assembly and cytokinesis triggers JNK pathway activation, upregulation of MMP1 and apoptotic cell death. Thus, our data suggest that Awd depletion leads to CIN.

Our previous studies on awd function already showed that this gene is required during development for normal wing development since adult flies, mosaic for awd [30] loss of function allele, show altered wing morphology. The analysis of en > awdi adults has shown that, in comparison with wild type (Fig. 1F), en > awdi wing discs develop into adult wings with a reduced posterior domain and bent toward the posterior (Fig. 1G, n = 20). This phenotype is consistent with the occurrence of apoptotic cell death induced by Awd depletion in larval wing disc.

**awd knockdown leads to abnormal eye differentiation.** We extended our analysis of awd gene function to the development of adult eye. The detailed analysis of the eye of adult flies expressing awdi in the larval eye disc under the control of the eyeless-Gal4 driver (ey > awdi) has showed aberrant morphological phenotype. In wild type adult eye about 700 ommatidia, each with an inter-ommatidial bristle, are organised in a highly regular array (Fig. 2A). On the contrary, ey > awdi adult eyes show clear defects in alignment of ommatidial facets and bristles and with some ommatidia missing (Fig. 2B). Furthermore, we have analysed the effects of awd [30] null mutation on eye development. Since this allele is lethal in homozygous condition, we have applied the directed mosaic technique to induce awd [30] mosaic clones in the eye disc. We have used the ey-flp line to target FRT/ Flp site specific recombination to the eye disc. Morphological analysis of ey-flp-induced awd [30] mosaic adult eyes also shows a rough eye phenotype results from loss of awd function (Fig. 2C). Our wing disc analysis show that knockdown as well as complete loss of awd function in larval wing and eye discs impairs normal development of the corresponding adult structures. Altered differentiation of adult structures arises from CIN induced aneuploidy in wing and eye discs, so our results on the role of awd in maintenance of genomic stability suggest that Awd knockdown leads to CIN.

**awd silencing leads to aneuploidy.** To avoid clearing of awd depleted cells, and better characterise the CIN events occurring in these cells, we have co-expressed awdi and p35 under control of en driver. Block of apoptotic machinery through expression of the effector caspase inhibitor p35 [23] leads in fact to persistence of CIN cells in tissues. These cells show altered plasma membrane polarity, defects in adherens junctions that connect cells and separate apical and basolateral cellular domains, and delamate from the epithelium. Then, we have analysed the effects of awdi and p35 co-expression on polarity of wing disc cells (hereafter referred as en > awdi, p35). We have focused on two proteins, the DE-cadherin (DE-Cad) component of adherens junctions and the atypical protein kinase C (aPKC) component of a-sub-apical protein complex. Wing discs were dissected from en > awdi, p35 larvae and in 10 out of 16 discs analysed we detected a visible reduction of DE-Cad (Fig. 3A) and aPKC levels (Fig. 3B). The differences in mean fluorescence intensity between GFP negative and GFP positive cells in each of the 16 wing discs have been analysed using a paired t-test. Applying this test the reduction of DE-Cad and aPKC stainings in GFP positive cells is statically significant (p = 0.0081, for DE-Cad and p = 0.0158, for aPKC) (Supplementary Data S1). Furthermore, en > awdi, p35 wing discs show strong up-regulation of MMP1 expression (Fig. 3C, n = 20). High levels of MMP1 lead to basement membrane degradation and cellular invasiveness in normal and tumoural tissues [19,25]. Interestingly, scanning across the vertical axis (x-z section in Fig. 3C) the
posterior region of en > awd, p35 wing discs shows that cells with high MMP1 level localise on the basal side of the epithelium. Thus, persistence of awd depleted cells in wing disc leads to CIN biological responses including delamination from the epithelium.
CIN consists of gain or loss of whole chromosomes or chromosomal regions leading to cellular aneuploidy. Thus, we have analysed the DNA content profile of cells from the posterior compartment of en > awdi, p35 wing discs using as control cells from the anterior compartment (Fig. 3D). Up to 38.5% of awdi cells has DNA content higher than 4n while only 11.3% control cells has a similar DNA content. Since it has been shown that wing disc cells expressing p35 have a percentage of cells with DNA content similar to wild type cells 8, these results clearly show that awdi cells are aneuploid.

Discussion
We report here, for the first time, a novel role for the Awd endocytic mediator in maintenance of genomic stability in larval disc epithelium. Our results show that depletion of Awd triggers JNK-mediated cell death of wing disc cells and that blocking the cell death machinery results in aneuploidy and cell delamination without overt hyperproliferative effect (Fig. 4). Overgrowth of wing disc hosting aneuploid cells is due to activation of the JNK pathway that promotes expression of Wingless (Wg) upon block of apoptotic cell death. Wg is a mitogenic molecule required in the imaginal discs for growth and patterning 26 and its expression in the aneuploid, delaminating CIN cells triggers growth of neighbouring non-delaminating cells 8. However, awd<sup>12A4</sup> mutant wing disc cells do not express Wg as consequence of faulty Notch signalling 13 therefore, they cannot promote hyperplasia of the surrounding tissue. Furthermore, lack of hyperproliferation is also observed when aneuploid condition arises
from impaired activity of genes controlling karyokinesis. The diaphanous gene (dia) codes for an actin-regulatory molecule which is required during acto-myosin driven contraction of metaphase furrows. Simultaneous depletion of dia gene expression and block of apoptosis do not lead to hyperplastic growth probably due to defective karyokinesis. Intriguingly, Awd is a microtubule-associated nucleoside diphosphate kinase that converts GDP to GTP and the analysis of awd mutant larval brain showed mitotic defects correlated with defective microtubule polymerisation. This raises the possibility that the Awd kinase function plays a role in GTP supply to protein such as Orbit which are required for stabilisation of spindle microtubules.

Two lines of evidence further support the hypothesis that Awd could be involved in karyokinesis. The first comes from studies showing that endosome trafficking and transport to the intercellular bridges of dividing cells plays a critical role during abscission, the last step of karyokinesis. In addition, remodelling of plasma membrane that underlies nuclear divisions in syncytial embryo and cellularisation also requires endocytosis. Embryo cellularisation requires the dynamin encoded by the shibire (shi) locus and Rab5 GTPase function since loss of function of either genes arrests progression of metaphase furrows. Awd functionally interacts with shi locus and it is also required for Rab5 function in early endosomes. Thus, a possible role for Awd in cytokinesis should be considered.

**Figure 3.** awd knockdown leads to CIN. (A–C) Confocal images of wing discs from en > awdi, p35 third instar larvae. Down-regulation of DE-Cad (A) and aPKC stainings (B) is detected in posterior compartment cells. To eliminate variability of image intensity among the different wing disc analysed, the mean fluorescence intensity value of GFP positive cells has been normalised to that in GFP negative cells. The histograms show the normalised fluorescence intensity for DE-Cad (A) and aPKC (B). In the same disc domain, the MMP1 staining (C) is greatly up-regulated and the cross section (x-z), along the position indicated by the yellow line, shows that these cells are delaminating. (D) Fluorescence associated cell sorter (FACS) of control (GFP−) and awd depleted cells (GFP+) from en > awdi, p35 wing discs. The dotted line outlines the anterior/posterior boundary (A,B). Scale bars: 5μm in (A,B), 25μm in (C).
The second line of evidence comes from studies on NME1, the human homolog of awd gene. This metastasis suppressor gene shares about 78% of amino acid identity with the awd gene. Down-regulation of NME1 gene expression in diploid cells results in cytokinesis failure and leads to tetraploidy. Our in vivo results show that Awd plays a role in maintenance of genomic stability confirming the high degree of conservation between NME1 and Awd proteins. Drosophila studies have already been crucial in identification of NME1 function in epithelial morphogenesis and our present work shows that it can be a useful model to investigate also this function and its impact on tumour development and progression.

Methods
Fly strains and husbandry. Stocks were raised on standard cornmeal/yeast/agar medium at 25°C, and crosses were carried out at the same temperature. We used the following Bloomington stocks: #5072, #5073, #30564, #33712, #5137. The stock ey-Gal4/TM6B was a gift from A. Giangrande (USIAS, Université de Strasbourg); awdJ2A4, FRT82B/TM6B (from T. Hsu, Boston University) and en-Gal4, UAS-GFPmCD8/CyO from D. Grifoni (University of Bologna). Larvae en-Gal4, UAS-GFPmCD8/+; UAS-awdRNAi and en-Gal4/UAS-P35; UAS-awdRNAi were obtained by crossing the parental strains.

Immunofluorescence Microscopy. Larval tissues were collected and treated at 110–120 hours after egg deposition. Larvae were dissected in 1xPBS at room temperature and fixed for 20 minutes in 4% formaldehyde and the immunostaining procedure was performed as previously described. After several washes in 1xPBS + 0.3% Triton X-100, wing imaginal discs were mounted on microscopy slides with Fluormount G. Subsequently, samples were analysed with TCS SL Leica confocal system. Digital images were assembled using the Adobe Photoshop software. The following primary antibodies were used: monoclonal mouse anti-ci 1:50 (DSHB) and anti-phosphoJNK 1:400 (Cell Signaling Technology) were detected with Cy3-conjugated goat anti-mouse 1:500 (Jackson); polyclonal rabbit anti-Awd 12 1:1000 was detected using Cy3-conjugated goat anti-rabbit 1:1000 (Jackson) or DyLight 647-conjugated goat anti-rabbit 1:500 (Jackson); rabbit anti-MMP1 1:50 (DSHB) was detected using Cy3-conjugated goat anti-rabbit 1:1000 (Jackson); anti-cleaved-Caspase3 1:100 (Cell Signaling Technology) was detected using Cy3-conjugated goat anti-rabbit 1:2000 (Sigma); anti-ξPKC 1:200 (Santa Cruz Biotechnology) was detected with Cy5-conjugated goat anti-rabbit 1:1000 (Jackson). DE-Cad 1:25 (DSHB) was detected using Cy3-conjugated goat anti-rat 1:1000 (Jackson).

Statistical analyses. Fluorescence stains have been captured on TCS SL Leica confocal microscope. Five stacks per wing disc have been acquired with zxy scaling of 630μm. Fluorescence intensity quantification of projected z-stack images has been restricted to neighbour areas of same size in anterior and posterior compartments. Image J software has been used for measurement of fluorescence intensity. Differences between mean fluorescence intensity have been estimated for statistical significance using a two-tailed distribution paired Student's t-test with Prism software.

Scanning Electron Microscopy. ey-Gal4/UAS-awdRNAi and ey-FLP; Act-Gal4, UAS-GFP; awdJ22A4, FRT82B/Tub-Gal80, FRT82B and control (ey-Gal4/UAS-GFP and ey-FLP; Act-Gal4, UAS-GFP; Tub-Gal80, FRT82B) flies were collected and washed several times in water and then dehydrated in 100% ethanol. Flies were then incubated in a solution of ethanol:tetramethylorthosilicate (TMOS) 1:1 for 2 hours and then let dry in TMOS 100% overnight at room temperature in a fume hood. The day after the heads were dissected and carefully mounted on an aluminium stub previously prepared with a double stick carbon tape. Samples were then coated with a gold film before SEM examination. Samples were analysed with SEM JEOL JSM-5400 microscope and images were recorded at accelerating voltage of 15kV.
Mounting of adult wings. The left wings from female flies were washed in 1xPBS, dehydrated in ethanol 100% and then dissected and mounted on glasses in lactic acid/ethanol (6:5). Wing images were captured using a Nikon Eclipse 90i microscope.

Flow cytometry analysis. Approximately 60 L3 wing discs for genotype were dissected in cold 1xPBS, centrifuged (5 minutes 3000rcf at RT) and dissociated into single cells in 0.05% Trypsin-EDTA (1X) (Gibco) for 4 h in RT. After dissociation, samples were incubated overnight at −20 °C in a solution of ethanol/PBS 2:5:1. After several washes in 1xPBS + EDTA, samples were incubated with propidium iodide, PI (15 minutes) and analysed by FACS.

PI fluorescence was determined by flow cytometry using a Fluorescent activated cell sorter BD FACsAria Cell Sorter (BD Biosciences). Excitation of the sample was carried out using a Coherent Sapphire Solid State laser. Excitation with 488 nm allowed the acquisition of forward-scatter (FS), side-scatter (SS), fluorescence from GFP. Doublets were discriminated using an integral/peak dot plot of PI fluorescence. Optical alignment was based on optimized signal from specialised fluorescent 6 μm particle (BD AccuDrop beads). DNA analysis on single fluorescence histograms was done using BD FACSDiVa software (BD Biosciences).

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Acknowledgements

We thank Roberta Randi for her help in performing SEM analyses and Catia Giovannini for technical assistance for FACS analysis. We thank Daniela Grifoni and Roberto Bernardoni and the *Drosophila* community for sharing reagents such as fly stocks and antibodies. We also thank Marilena Ignetti for critical reading of the manuscript and helpful suggestions. We thank Marco Privitera for his graphic work. The authors gratefully acknowledge funding from the Association for International Cancer Research, AICR-WWCR grant ref. 11-0738, to V. Cavaliere and from the University of Bologna (RFO 2013/14) to G. Gargiulo and V. Cavaliere. This work was also supported by the Associazione Italiana per la Ricerca sul Cancro-AIRC, grant “MFAG-16491”, to S. Duchi. The AICR-WWCR funds have been used to pay for a research grant awarded from the University of Bologna to P. Romani.

Author Contributions

P.R. performed *Drosophila* experimental work and S.D. performed FACS analysis. P.R., G.G. and V.C. participated in the discussion and designed the experiments. V.C. wrote the manuscript. All authors read and approved the final manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-17217-0.

Competing Interests: The authors declare that they have no competing interests.

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