ABSTRACT
The deregulation of cell polarity or cytoskeletal regulators is a common occurrence in human epithelial cancers. Moreover, there is accumulating evidence in human epithelial cancer that BTB-ZF genes, such as Bcl6 and ZBTB7A, are oncogenic. From our previous studies in the vinegar fly, Drosophila melanogaster, we have identified a cooperative interaction between a mutation in the apico-basal cell polarity regulator Scribble (Scrib) and overexpression of the BTB-ZF protein Abrupt (Ab). Herein, we show that co-expression of ab with actin cytoskeletal regulators, RhoGEF2 or Src64B, in the developing eye-antennal epithelial tissue results in the formation of overgrown amorphous tumours, whereas ab and DRac1 co-expression leads to non-cell autonomous overgrowth. Together with ab, these genes affect the expression of differentiation genes, resulting in tumours locked in a progenitor cell fate. Finally, we show that the expression of two mammalian genes related to ab, Bcl6 and ZBTB7A, which are oncogenes in mammalian epithelial cancers, significantly correlate with the upregulation of cytoskeletal genes or downregulation of apico-basal cell polarity neoplastic tumour suppressor genes in colorectal, lung and other human epithelial cancers. Altogether, this analysis has revealed that upregulation of cytoskeletal regulators cooperate with Abrupt in Drosophila epithelial tumorigenesis, and that high expression of human BTB-ZF genes, Bcl6 and ZBTB7A, shows significant correlations with cytoskeletal and cell polarity gene expression in specific epithelial tumour types. This highlights the need for further investigation of the cooperation between these genes in mammalian systems.

KEY WORDS: Drosophila, Eye-antennal disc, Apico-basal cell polarity, Actin cytoskeletal regulators, BTB-ZF, Abrupt, RhoGEF2, Rac1, Src, Scribble

INTRODUCTION
Cancer is a cooperative process involving many mutations that lead to the deregulation of the normal controls that regulate cell proliferation, survival, differentiation and migration, amongst other processes (Hanahan and Weinberg, 2011). Understanding the molecular events that occur during cooperative tumorigenesis is critical in order to develop therapeutics to combat cancer. The model organism, Drosophila melanogaster (vinegar fly), has proven to be an excellent model for the discovery of new tumorigenic genes and the dissection of their roles in tumour progression, and has proven relevance to human cancer (Brumby and Richardson, 2005; Cheng et al., 2013; Gonzalez, 2013; Rudrapatna et al., 2012; Stefanatos and Vidal, 2011).

Recently, the disruption of apical-basal cell polarity, which affects cell adhesion and signalling pathways and leads to an epithelial to mesenchymal transition (EMT), has been realized as a new hallmark of cancer (Elsum et al., 2012; Hanahan and Weinberg, 2011; Humbert et al., 2008). Central to cell polarity regulation are the Scribble module (Scribble (Scrib), Dlg and Lgl), the Crumbs module (Crumbs, Pals and Patj) and the Par module (Par6, Par3 and aPKC), which undergo positive or negative interactions in the establishment and maintenance of the apical and basolateral domains of an epithelial cell. Whole organism or tissue-specific depletion of genes in the Scribble module lead to a loss of cell polarity and aberrant signalling, leading to the formation of neoplastic tissue in Drosophila epithelial tissues (Elsum et al., 2012; Humbert et al., 2008). However, when scrib, dlg or lgl are mutated in patches of cells within the developing eye-antennal tissue, despite deregulation of signalling pathways and cell proliferation, tissue overgrowth does not ensue due to cell differentiation and Jun N-terminal Kinase (JNK)-mediated apoptosis (Brumby and Richardson, 2003; Doggett et al., 2011; Grzeschik et al., 2007, 2010; Igaki et al., 2006; Uhlirova and Bohmann, 2006; Uhlirova et al., 2005).

In investigating cooperation between polarity loss and oncogenic pathways in epithelial tumorigenesis, we discovered that activation of the small GTPase, Ras (Ras85D) (herein) or activated Notch (NotchICD, referred to as NotchΔE7 herein) cooperated with scrib loss-of-function to form massive invasive tumours (Brumby and Richardson, 2003). Subsequent analysis showed that cooperation depended upon activation of the JNK and downregulation of the Hippo negative tissue growth control pathways, thereby promoting tumour growth, inhibiting differentiation and promoting an invasive phenotype (Doggett et al., 2011; Igaki et al., 2006; Leong et al., 2009; Uhlirova and Bohmann, 2006; Uhlirova et al., 2005). This mechanism is conserved in mammalian cells and mouse models, where depletion or knockout of scrib leads to hyperplasia, and additional expression of the Ras oncogene (Ha-RasΔ12) cooperates with scrib loss-of-function to promote tumorigenesis (Dow et al., 2008; Elsum et al., 2013; Godde et al., 2014; Pearson et al., 2011). Moreover, similar to that observed in Drosophila, the expression of JNK is able to cooperate with...
Rac1 also contribute to its cooperative effects with RasACT (Myat, 2010). It is therefore likely that these downstream effects of Ha-RasV12 phenotype and also resulted in morphological and differentiation clones act similarly to screen for genes that when over-expressed in eye-antennal disc (Brumby et al., 2011). DRac1 showed neoplastic growth in cooperation with E-cadherin and regulators, Crumbs and aPKC, and the adherens junction proteins (EMT) by mislocalization or loss of expression of the apical polarity morphology, resembling an epithelial to mesenchymal transition Drosophila (Brumby et al., 2011). DRac1 abrupt loss-of-function (Turkel et al., 2013). In this screen, we identified abrupt (ab), which in cooperation with scrib loss-of-function promotes the retention of a progenitor-like cell state by blocking expression of differentiation genes, as well as promoting tumour growth and invasion. Abrupt encodes a Broad-Complex, Tramtrack, Bric-a-brac domain (BTB)-zinc-finger (ZF) transcription factor with roles in neuromuscular junction and dendrite morphogenesis, ovarian border cell migration and imaginal disc epithelial development (Grieder et al., 2007; Hattori et al., 2013; Hu et al., 1995; Jang et al., 2009). BTB-ZF transcription factors are a large family of proteins, with 47 human members, many of which have been shown to be associated with cancer (Costoya, 2007; Kelly and Daniel, 2006). The most well known of the BTB-ZF mammalian family members are Bcl6 and ZBTB7 (LRF/Pokemon), which function as proto-oncogenes in lymphomas, leukemias and solid cancers (Hatzli and Melnick, 2014; Maeda et al., 2005). In solid cancers, Bcl6 is upregulated in breast, colorectal and squamous head and neck epithelial cancers, and contributes to their growth and progression (Sen et al., 2014; Walker et al., 2014; Worsham et al., 2012; Wu et al., 2014). ZBTB7A is upregulated in colorectal, bladder, breast, prostate, non-small cell lung cancer and liver cancers and reducing its expression blocks tumour development (Aggarwal et al., 2010, 2011; Guo et al., 2014; Leon et al., 2008; Liu et al., 2012; Qu et al., 2010; Zhang et al., 2013; Zhao et al., 2013, 2008).

In a Drosophila genetic screen for Ras-cooperating genes (using ey>RasACT, where expression of RasACT is driven via the eyeless promoter throughout the developing eye), we identified the actin cytoskeletal regulatory genes, RhoGEF2 and DRac1 (Brumby et al., 2011). These genes enhanced the ey>RasACT hyperplastic adult eye phenotype and also resulted in morphological and differentiation defects (Brumby et al., 2011). Furthermore, RhoGEF2 and DRac1 showed neoplastic growth in cooperation with RasACT in a clonal context in the eye-antennal disc (Brumby et al., 2011). DRac1 (Drosophila Rac1) is a member of Rho/Rac/Cdc42 small-GTPase superfamily, key regulators of the actin cytoskeleton (Jaffe and Hall, 2005; Szczepanowska, 2009), and is involved in morphological cell shape changes during Drosophila development (Harden et al., 1995; Settlemann, 1999; Van Aelst and D’Souza-Schorey, 1997). Indeed, constitutive activation of Rac1 during tube morphogenesis of the Drosophila salivary gland causes changes in epithelial cell morphology, resembling an epithelial to mesenchymal transition (EMT) by mislocalization or loss of expression of the apical polarity regulators, Crumbs and aPKC, and the adherens junction proteins E-cadherin and β-catenin (Pirriglia et al., 2006; Pirriglia and Myat, 2010). It is therefore likely that these downstream effects of Rac1 also contribute to its cooperative effects with RasACT in tumorigenesis in the eye-antennal disc (Brumby et al., 2011).

RhoGEF2 is a guanine nucleotide exchange factor (GEF) (Schmidt and Hall, 2002) that acts via activating the small GTPase, Rho1, in morphological cell shape changes during Drosophila development (Barrett et al., 1997; Häcker and Perrimon, 1998; Mulinar et al., 2008; Nikolaidou and Barrett, 2004; Padash Barmchi et al., 2005; Rogers et al., 2004). Consistent with RhoGEF2 functioning via Rho1, we also found that an activated allele of Rho1 (RhoV14) was also a RasACT cooperating oncogene in epithelial tumorigenesis (Brumby et al., 2011). RhoGEF2 cooperates with RasACT in tumorigenesis by activating the Rho1-Rok-MyoII-JNK pathway (Khoo et al., 2013). Interestingly, MyoII activity (pMRLC) is increased in scrib− RasACT eye-antennal disc clones and contributes to scrib RasACT tumorigenesis (Kulshammer and Uhlirova, 2013), as does JNK activation (Igaki et al., 2006; Leong et al., 2009). Uhlirova and Bohmann, 2006).

Furthermore, in this genetic screen, we identified another cytoskeletal regulator, Src-42A, a Drosophila homolog of the Src tyrosine kinase (Thomas and Brugge, 1996), but were unable to confirm its cooperative interaction with RasACT with an independent transgene (Brumby et al., 2011). However, we found that overexpression of the second Drosophila Src family member, Src-64B, using a transgenic line (Dodson et al., 1998), showed strong cooperation with RasACT when expressed globally in the developing eye or in eye-antennal disc MARCM clones (C.P., A.B., H.R., unpublished data). Src-64B also functions in regulation of the actin cytoskeleton and cell shape changes during development in Drosophila (Dodson et al., 1998; Guarneri et al., 1998; Kelso et al., 2002; O’reilly et al., 2006; Roulier et al., 1998; Strong and Thomas, 2011; Takahashi et al., 1996). Depending on the context, upregulation of Src-64B or Src-42A activity (via overexpression of the Src genes or Csk downregulation) can lead to either increased proliferation, or apoptosis and invasion (Pedraza et al., 2004; Read et al., 2004; Vidal et al., 2006, 2007). Recent studies have also shown that overexpression of Src-42A or Src-64B in Drosophila adult intestinal progenitor cells results in progenitor cell over-proliferation (Cordero et al., 2014; Kohlmaier et al., 2014). Furthermore, in the developing wing epithelium blocking apoptosis in tissues expressing Src-64B results in overgrowth (Fernández et al., 2014), and in the eye-antennal epithelium Src-64B or Src-42A upregulation (or downregulation of the Src negative regulator, Csk) cooperates with RasACT to result in neoplastic tumour formation (Enomoto and Igaki, 2013; Vidal et al., 2010, 2007).

Since RhoGEF2, DRac1 or Src are cooperating oncogenes with RasACT, and ab overexpression phenocopies RasACT or NotchACT in cooperative tumorigenesis with scrib loss-of-function (Turkel et al., 2013), we sought to determine whether ab could also cooperate with RhoGEF2, DRac1 or Src-64B in tumorigenesis. Herein, we describe the effect of co-expression of ab with RhoGEF2, DRac1 or Src-64B in the developing eye-antennal epithelium. We show that co-expression of ab with RhoGEF2 or Src-64B results in neoplastic tumour formation, whereas ab and DRac1 co-expression leads to non-cell autonomous overgrowth. We show that together with ab these genes affect the expression of differentiation genes. Finally, we investigate whether the expression of two mammalian genes related to ab, Bcl-6 and ZBTB7A, which are oncogenic in mammalian cancer, are correlated with the upregulation of cytoskeletal genes or downregulation of apico-basal cell polarity neoplastic tumour suppressor genes in human epithelial cancers.

**RESULTS**

**Cooperation of abrupt with RhoGEF2**

To determine if ab cooperates with Ras-cooperative oncogene, RhoGEF2, to drive tumorigenesis, we generated clones expressing ab and RhoGEF2 using the MARCM system (Lee and Luo, 1999), and compared tumour development to scrib ab-expressing clones in the Drosophila developing eye-antennal epithelium (Fig. 1). Our previous studies have shown that the overexpression of ab in otherwise wild-type eye disc clones promoted antennal disc overgrowth, but did not block photoreceptor differentiation. Mutation of scrib alone in clones results in cell morphology changes and disorganisation, but does not dramatically affect

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differentiation as revealed by Elav staining or lead to tissue overgrowth and larvae enter pupariation normally at day 5/6 after egg deposition (AED) (Brumby and Richardson, 2003; Turkel et al., 2013). However, scrib−/−ab-expressing clones (marked by GFP) overgrow at the expense of the surrounding normal tissue (GFP negative) over an extended larval period and form massive tumours (Fig. 1A1,B2), which fuse with the surrounding tissue and invade into the brain (Turkel et al., 2013), similar to that observed for scrib−/−RasACT tumours (Brumby and Richardson, 2003). scrib−/−ab-expressing clones showed cell morphology defects, as revealed by F-actin staining (Fig. 1A2,B2), and an inhibition of photoreceptor cell differentiation in the eye epithelium, as revealed by Elav staining (arrowheads, Fig. 1A1-A4).

RhoGEF2 expression in mosaic disc produced small clones with increased F-actin levels, and cell morphology and differentiation defects (arrowheads, Fig. 1C1,C3,C4) (Brumby et al., 2011; Khoo et al., 2013). At day 5/6 AED ab RhoGEF2 co-expressing clones were smaller than the surrounding wild-type clones (Fig. 1D1,D4) and accumulated F-actin (Fig. 1D2). ab RhoGEF2 mosaic discs also showed non-cell autonomous effects, as the surrounding wild-type
tissue exhibited folding and distortion around the clonal tissue at day 5 (Fig. 1D2). At day 8/9 AED, *ab RhoGEF2* eye disc clones were overgrown relative to wild-type tissue (Fig. 1E), although folded wild-type tissue was present around clonal tissue. However, antennal disc clones did not overgrow and remained a similar size as day 5 clones. Differentiation as marked by Elav was reduced in eye disc clones throughout larval development (arrowheads, Fig. 1D1-D4,E1-E4). The effect of *ab RhoGEF2* cooperation led to a failure in pupation and the formation of giant larvae (not shown), similar to *RhoGEF2 RasACT* cooperation (Khoo et al., 2013). However, in comparison to *scrib−ab* tumours, which exhibit fusion of the two eye-antennal discs that is associated with an invasive phenotype (Turkel et al., 2013), *ab RhoGEF2* did not show strong invasive properties, since the two eye-antennal discs did not fuse together (data not shown). Indeed, the cooperative tumorigenic effect of *ab RhoGEF2* was most similar to *RasACT RhoGEF2* cooperation, with the exception of the effect on the antennal disc (Brumby et al., 2011; Khoo et al., 2013). Taken together, these data show that *RhoGEF2* is capable of cooperating with *ab* to produce overgrown, undifferentiated and amorphous tumours.

**Cooperation of abrupt with Src64B**

Since Src64B can cooperate with RasACT (see introduction), we wished to determine if *ab* also cooperates with Src64B. When expressed alone, Src64B resulted in large clones in the antennal and the anterior portion of the eye disc, which showed high levels of F-actin accumulation (Fig. 2A2). Clones in the posterior differentiated region of the eye disc proper were very small and did not noticeably affect differentiation, although larger clones were observed in the overlying peripodial layer leading to the displacement of the underlying differentiated tissue (Fig. 2A1,A3,A4). Src64B-expressing mosaic larvae pupated normally, but were delayed in development and eclosed 1–2 days after their control counterparts (not shown). Co-expression of *Src64B* and *ab* resulted in large clones in the antenna and the eye discs, including the posterior region of the eye disc at day 5/6 AED (Fig. 2B1), however these were not significantly overgrown relative to the wild-type tissue. However at day 8/9 AED, *Src64B ab* eye disc clones were clearly overgrown relative to wild-type tissue. *Src64B ab* co-expressing clones had rounded-edges with high levels of F-actin at day 5/6 and day 8 AED (Fig. 2B2,C2). Differentiation, as revealed by Elav staining, was abolished in clones in the posterior region of the eye disc (arrowheads, Fig. 2B1-B4,C1-C4). The overall size of Day 8/9 *Src64B ab* mosaic eye-antennal discs were overgrown relative to wild-type mosaic eye-antennal discs, however there was slightly more wild-type tissue remaining at day 8/9 AED compared to *scrib−ab* mosaic discs (compare Fig. 2C with Fig. 1B). *Src64B ab* cooperation led to the formation of giant larvae and a failure of pupation (not shown), however they did not result in the fusion of the two eye-antennal discs (not shown), as occurs with *scrib−ab* tumours. Altogether, these data indicates that *ab* cooperates with Src64B to promote overgrown, undifferentiated and amorphous tumours.

**Cooperation of abrupt with DRac1**

Since DRac1 and RasACT cooperate to form invasive tumours in the eye-antennal epithelium (Brumby et al., 2011), we sought to investigate if *ab* and DRac1 also cooperate in tumorigenesis.

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![Fig. 2. Src64B cooperates with ab to form large tumours.](image-url)

Confocal planar images of mosaic larval eye-antennal discs stained for F-actin (with Phalloidin, grey or red in merge) and Elav (grey or blue in merge); mutant clones are GFP+ and wild-type tissue is GFP− (grey or green in merge). (A) *Src64B* mosaic eye-antennal disc at day 5 AED. (B) *ab Src64B* mosaic eye-antennal disc at day 5 AED. (C) *ab Src64B* mosaic eye-antennal disc at day 8 AED. Arrowheads point to patches of mutant tissue showing differentiation defects. Genotypes: (A) *ey-FLP, UAS-GFP; UAS-Src64B; FRT82B/tubGAL4; FRT82B, tubGAL80* (B,C) *ey-FLP, UAS-GFP; UAS-Src64B; UAS-ab56, FRT82B/tubGAL4; FRT82B, tubGAL80.* Scale bars=50 μM.
In mosaic eye-antennal discs at day 5 AED, DRac1 over-expression produced small clones with cell morphology defects (although F-actin levels were only slightly increased, Fig. 3A2) and disrupted Elav expression (yellow arrowheads, Fig. 3A1-A4). Over-expression of ab with DRac1 resulted in large clones mostly in the anterior region of the eye disc (Fig. 3B), although overall there was less mutant clonal tissue in the eye-antennal disc compared with the wild-type mosaic eye-antennal disc. At later times (day 8/9 AED), DRac1 ab co-expression resulted in strong non-cell autonomous effects, as indicated by the highly folded wild-type tissue surrounding the clonal tissue and greater representation of GFP− tissue (Fig. 3C). Over-expression of ab with DRac1 resulted in large clones mostly in the anterior region of the eye disc (Fig. 3B), although overall there was less mutant clonal tissue in the eye-antennal disc compared with the wild-type mosaic eye-antennal disc. At later times (day 8/9 AED), DRac1 ab co-expression resulted in strong non-cell autonomous effects, as indicated by the highly folded wild-type tissue surrounding the clonal tissue and greater representation of GFP− tissue (Fig. 3C).

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**Comparison of cooperative interactions relative to scrib− ab tumours**

The comparative overgrowth at day 5/6 AED and day 8/9 AED for expression of the actin cytoskeletal genes with ab relative to scrib− ab is summarized in Fig. 4. To determine the relative overgrowth of the mutant tissue to wild-type tissue we quantified the volume of GFP+ tissue to total eye-antennal disc volume for all genotypes at day 5/6 and day 8/9 (Fig. 4A,B). At day 5/6 AED the GFP+ tumour volume relative to the total disc volume for RhoGEF2 ab, Src64B ab and DRac1 ab was similar to the FRT control, but scrib− ab clonal tissue was slightly reduced relative to wild-type tissue (Fig. 4A). However, at day 8/9 AED, scrib− ab GFP+ tumours represented the

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**Fig. 3. Co-expression of DRac1 with ab results in non-cell autonomous overgrowth.** Confocal planar images of mosaic larval eye-antennal discs stained for F-actin (with Phalloidin, grey or red in merge) and Elav (grey or blue in merge); mutant clones are GFP+ and wild-type tissue is GFP− (grey or green in merge). (A) DRac1 mosaic eye-antennal disc at day 5 AED. (B) ab DRac1 mosaic eye-antennal disc at day 5 AED. (C) ab DRac1 mosaic eye-antennal disc at day 8 AED. (D1) ab DRac1 escaper adult fly heads, side and dorsal views. (D2) wild-type (control) adult fly heads, side and dorsal views. Yellow arrowheads point to patches of mutant tissue showing differentiation defects. Note that in panel C differentiation was observed in the wild-type tissue in the posterior region of the eye disc, but the highly folded nature of the wild-type tissue makes this difficult to image in a single Z section. White arrowheads point to an example of elevated F-actin. Genotypes: (A) ey-FLP, UAS-GFP; UAS-DRac1; FRT82B/tubGAL4; FRT82B, tubGAL80. (B,C,D1) ey-FLP, UAS-GFP; UAS-DRac1; UAS-ab56, FRT82B/tubGAL4; FRT82B, tubGAL80. (D2) ey-GAL4. Scale bars=50 μM.
In order to assess how death affects tissue growth via effects on cell proliferation and cell differentiation of photoreceptor development as judged by ELAV staining (Fig. 4C). Relative to scrib− ab cooperative tumorigenesis, co-expression of the cytoskeletal genes with ab resulted in less potent cooperative overgrowth at day 8/9 AED (Fig. 4C).

**Cooperation of abrupt with RhoGEF2, Src64B or DRac1 affects tissue growth via effects on cell proliferation and cell death**

In order to assess how ab was cooperating with RhoGEF2, Src64B or DRac1 relative to scrib− to affect tissue growth, we performed EdU labelling to reveal proliferating cells and TUNEL labelling to detect dying cells in mosaic eye-antennal discs from all genotypes at day 5/6 and day 8/9 (Figs 5 and 6). The EdU labelling experiment revealed that relative to the FRT control where cell proliferation ceases in the posterior region of the eye disc (Fig. 5A), scrib− ab, RhoGEF2 ab, and Src64B ab GFP+ clones showed increased numbers of EdU+ cells in the posterior region as well as throughout the eye-antennal discs at day 5 and day 8 AED (Fig. 5B,C,F-J), however DRac1 ab GFP+ clones showed a reduction in EdU incorporation relative to the surrounding wild-type tissue (Fig. 5D,E,J). The analysis of cell death, revealed that there were more dying cells in the wild-type tissue (GFP−) in scrib− ab, RhoGEF2 ab, and Src64B ab mosaic discs at day 5 and day 8 AED (Fig. 6B,C,F-J) versus the FRT control that showed only low levels of TUNEL+ cells (Fig. 6A). Conversely, DRac1 ab GFP+ clones showed more dying cells relative to the wild-type tissue at day 8 AED (Fig. 6E,J), although similarly low numbers of TUNEL+ cells were present in the mutant tissue (GFP+) versus wild-type tissue (GFP−) at day 5 AED (Fig. 6D,J). Altogether, these results show that increased cell proliferation of the mutant tissue and increased cell death of the wild-type tissue occurs in scrib− ab, RhoGEF2 ab, and Src64B ab mosaic discs, whilst the opposite occurs in DRac1 ab mosaic discs. The EdU and TUNEL patterns are generally consistent with the tissue overgrowth data at day 8 (Fig. 4B), with the exception of RhoGEF2 ab, where the tumour did not overgrow relative to the wild-type tissue. Since EdU measures S phase cells, it is possible there might be delays in G2/M phase in the mutant tissue in this genotype to account for this effect. At day 5, the tumour volume was similar to wild-type for all samples, except for scrib− ab where mutant tissue was less represented (Fig. 4A), therefore the EdU and TUNEL data at day 5 does not reflect tumour volume at this stage, but predicts what occurs later in tumour development (i.e. day 8).

**Cooperation of abrupt with RhoGEF2, Src64B or DRac1 affects expression of critical eye and antennal differentiation genes**

We have previously shown, by ChIP sequencing of Ab targets and expression array analysis, that Ab regulates the expression of eye-antennal cell fate genes and that this effect is enhanced or altered in ab scrib− tumours (Turkel et al., 2013). Since co-expression of ab with RhoGEF2, Src64B or DRac1 also affects expression of the eye differentiation factor, Elav, we sought to determine whether other cell fate genes in eye and antennal development...
were also affected in these tumours. In eye development, Dachshund (Dac) is one of the earliest transcriptional regulators that drives cell fate determination in the developing eye (Chen et al., 1997; Shen and Mardon, 1997), and expression of Dac is blocked in ab scrib−tumours (Turkel et al., 2013). We therefore wished to determine if this was also the case in ab cytoskeletal gene cooperative tumours.

In wild-type eye-antennal discs, Dac is expressed in a broad band in the middle of the eye disc and also in a crescent in the antennal disc (Fig. 7A). scrib1 ab clones do not express Dac in the eye disc (arrowheads, Fig. 7B1-B4) or in the antennal disc. Dac expression is only slightly reduced in scrib1 clones and unaffected in ab overexpressing clones in the eye disc (Turkel et al., 2013). In RhoGEF2 ab clones in the eye disc, Dac expression was blocked (arrowheads, Fig. 7C1-C4). Similarly, Dac expression was blocked in Src64B ab clones (arrowheads, Fig. 7D1-D4) and in DRac1 ab clones (arrowheads, Fig. 5E1-E4). Dac expression was also blocked in the antennal disc in ab RhoGEF2, ab Src64B or ab DRac1 co-expressing clones (Fig. 7C-E; data not shown). Thus, similarly to ab scrib−tumours, ab cytoskeletal gene tumours appear to be blocked in differentiation prior to Dac expression.

In antennal disc differentiation, initial expression domains of the transcription factors Homothorax (Hth), Cut (Ct) and Distal-less (Dll) during 2nd instar larval development establish the early proximo-distal axis of the antenna (Dominguez and Casares, 2005). We have previously shown that scrib− ab clones retain the expression of Dll within the growing tumour, but downstream regulated genes, such as Dac, are not retained (Turkel et al., 2013). We therefore tested if Dll was still expressed in ab cytoskeletal gene tumours.

In wild-type antennal discs, Dll is expressed in more distally destined cells in the antennae (Fig. 8A), and scrib− ab clones retain this expression (arrowheads, Fig. 8B1-B4). Expression of RhoGEF2 with ab did not block Dll expression, and instead an enlarged Dll-expression domain was observed (arrowheads,
Fig. 8C1-C4), probably due to a partial duplication of the antennae, which is sometimes observed in ab-expressing clones (Turkel et al., 2013). Surprisingly, Src64B ab clones showed reduced expression of Dll (arrowheads, Fig. 8D1-D4) and distortion of the antennal structures due to cell morphology changes (Fig. 8D2). In DRac1 ab clones, normal expression of Dll was also observed (arrowheads, Fig. 8E1-E4). Altogether, these results show that RhoGEF2 ab and DRac1 ab are similar to scrib− in cell fate status, however Src64B ab tumours are blocked at an earlier progenitor cell state than scrib− tumours (summarized in Fig. 9).

Correlation in expression of oncogenic BTB-Zn finger genes, Bcl6 and ZBTB7A, with apico-basal cell polarity and cytoskeletal genes in human epithelial cancer

Since we have shown here that ab cooperates with the cytoskeletal regulators, RhoGEF2 and Src64B, to result in cooperative tumorigenesis, we wished to determine whether the expression of human homologs of these genes showed cooperation with BTB-Zn finger genes in human cancers. Since our previous studies had also shown that the cell polarity tumour suppressor, scrib, showed cooperative tumorigenesis with ab (Turkel et al., 2013), we also sought to determine whether human homologs of the Scribble module were downregulated in human tumours, showing high expression of BTB-Zn finger genes. Furthermore, since we have shown that the JNK signalling pathway was important in the invasive properties of these tumours and sufficient to cooperate with RasACT in Drosophila and mammalian invasive tumour growth (Brumby et al., 2011), we wished to examine the correlation of expression of the human JNKK and JNK homologs with BTB-Zn finger genes in human cancer. Of the human BTB-Zn finger genes, there is greatest evidence for Bcl6 and ZBTB7A as oncogenes in human epithelial cancer (see Introduction), so we focused our analysis on these genes. Using Oncomine, we analysed collections of human epithelial cancers for expression correlation with Bcl6 or ZBTB7A and human RhoGEF2.
homologs (ARHGEF1, ARHGEF11, ARHGEF12), Src homologs (Src, Yes, Fyn), Scribble module genes (hscrib, lgl1, lgl2, dlg1, dlg2, dlg3, dlg4), JNKK homologs (MAP2K4, MAP2K7) and JNK homologs (MAPK8, MAPK9, MAPK10). In this analysis, the cancer samples were compared with normal tissues where available. The correlation of expression of each of these polarity and cytoskeletal genes in each dataset revealed that there were significant correlations (P<0.05) for several cytoskeletal or polarity regulatory genes with Bcl6 or ZBTB7A in several cancer types (Table 1; supplementary material Table S1 and supplementary data). Most interestingly, in the Gaspar Colon colorectal adenoma dataset (Gaspar et al., 2008) Bcl6 expression was significantly positively correlated with MAP2K4, Yes1 and negatively correlated with Dlg2 and Lgl1 (Fig. 10B, Table 1). Stratification of the Rohrbeck Lung cancers dataset into different stages showed that there were several samples of lung adenocarcinoma or lung squamous cell carcinoma having high Bcl6 expression and high MAP2K4 expression relative to normal lung (No value), whereas high Bcl6 expression correlated with low Dlg2 or Ltg1 in some samples from all forms of lung cancers relative to normal lung (Fig. 10B). Also significantly positively correlated with a stronger trend compared with normal tissue were Bcl6 and MAP2K7 in the Boersma breast epithelial cancer (Boersma et al., 2008) and ZBTB7A and MAP2K7 in the Zhai cervical squamous carcinoma dataset (Zhai et al., 2007) (Table 1). Also significant was that in the Toruner Head-Neck all oral squamous carcinoma (cancer only) dataset (Rohrbeck et al., 2008) Bcl6 expression was positively correlated with MAP2K4, Yes1 and negatively correlated with Dlg2 and Lgl1 (Fig. 10B, Table 1). Stratification of the Rohrbeck Lung cancers dataset into different stages showed that there were several samples of lung adenocarcinoma or lung squamous cell carcinoma having high Bcl6 expression and high MAP2K4 expression relative to normal lung (No value), whereas high Bcl6 expression correlated with low Dlg2 or Ltg1 in some samples from all forms of lung cancers relative to normal lung (Fig. 10B). Also significantly positively correlated with a stronger trend compared with normal tissue were Bcl6 and MAP2K7 in the Boersma breast epithelial cancer dataset (Boersma et al., 2008) and ZBTB7A and MAP2K7 in the Zhai cervical squamous carcinoma dataset (Zhai et al., 2007) (Table 1). Also significant was that in the Toruner Head-Neck all oral squamous carcinoma (cancer only) dataset (Toruner et al., 2004) positive correlations were seen between Bcl6 and MAPK10 and between ZBTB7A and ArhGef12, and in the Tomlins prostate carcinoma
dataset (Tomlins et al., 2007) Bcl6 expression was positively correlated with ArhGef11 and MAPK8 (Table 1). Significant positive correlations were also observed in the Collisson Pancreatic adenocarcinoma (cancer only) dataset (Collisson et al., 2011) between ZBTB7A and Src (Table 1). Furthermore, in the Gruitzmann pancreatic ductal adenocarcinoma dataset (Gruitzmann et al., 2004), although of borderline significance, a positive correlation was observed between ZBTB7A and MAP2K7 that showed a stronger trend compared with normal tissue (Table 1). Thus, taken together, these data show that in certain epithelial cancers the upregulation of Bcl6 or ZBTB7A expression is significantly correlated with reduced expression of Dlg2 or Llgl1 cell polarity genes or high expression of ArhGef11, ArhGef12, MAP2K4, MAP2K7, MAPK8, MAPK9, MAPK10, Src or Yes1 cytoskeletal genes. Based on our functional data in Drosophila and mammalian cells (this study; Brumby et al., 2011; Khoo et al., 2013; Turkel et al., 2013; C.P., A.B., H.R., unpublished data), we would expect the concordant expression of Bcl6 or ZBTB7A with these genes should result in tumour growth, morphology changes, differentiation blockage and invasive properties.

DISCUSSION

In this study, we have shown that over-expression of the Ab BTB-ZF protein cooperates with upregulation of RhoGef2 or Src64B in tumorigenesis, whereas Ab and DRac1 do not cooperate. Furthermore, we show that expression of Ab with each of these cytoskeletal regulators results in disruption to differentiation, in that the photoreceptor cell marker, Elav, and the early cell fate gene, Dac, are not expressed, although the antennal cell fate gene, Dll, is retained in all except ab Src64B co-expressing clones. Finally, we have found significant correlations in human epithelial cancer datasets between the high expression of BTB-ZF oncogenes, Bcl6 and ZBTB7A, and low expression of Dlg2 or Llgl1 cell polarity genes or high expression of ArhGef11, ArhGef12, MAP2K4,
MAP2K7, MAPK8, MAPK9, MAPK10, Src or Yes1 cytoskeletal genes. This data suggests that cooperation between these genes may occur in some human epithelial cancers.

**Comparison of tumorigenic properties**

RhoGEF2 ab or Src64B ab tumours showed overgrowth during an extended larval period resulting in giant larvae and loss of differentiation (Fig. 4C). However, unlike scrib− ab tumours there was also non-cell autonomous proliferation and the tumours did not appear to be as invasive as scrib− ab tumours, although a more detailed analysis of this is required. By contrast, co-expression of DRac1 and ab did not result in cooperative tumorigenesis, but rather non-cell autonomous proliferation. Relative to the cooperation of these cytoskeletal genes with RasV12 (Brumby et al., 2011; Khoo

**Table 1. Significant correlations in gene expression**

| Gene expression datasets | BTB-Zn gene | Polarity or cytoskeletal gene | P value | Pearson R | Notes |
|--------------------------|-------------|-------------------------------|---------|-----------|-------|
| **Positive Correlations** |             |                               |         |           |       |
| Tomlins Prostate all     | Bcl6        | ARHGEF11                      | 5.60×10⁻³ | 0.45      | A     |
| Tomlins Prostate Carcinoma | Bcl6    | ARHGEF11                      | 1.40×10⁻² | 0.57      | *, #  |
| Toruner Head-Neck all oral squamous carcinoma (cancer only) | Bcl6 | ARHGEF12                      | 1.70×10⁻² | 0.59      | *     |
| Toruner Head-Neck all oral squamous carcinoma (cancer only) | Bcl6 | MAPK10                        | 1.10×10⁻² | 0.62      | *     |
| Tomlins Prostate all     | Bcl6        | MAPK8                         | 2.00×10⁻² | 0.46      | A     |
| Tomlins Prostate Carcinoma | Bcl6    | MAPK8                         | 2.20×10⁻² | 0.57      | *     |
| Gaspar colon all         | Bcl6        | MAPK9                         | 3.20×10⁻¹³ | 0.71      | A     |
| Gaspar colorectal adenoma | Bcl6    | MAPK9                         | 4.40×10⁻⁹ | 0.69      | *, #  |
| Gaspar colon normal      | Bcl6        | MAPK9                         | 3.10×10⁻⁸ | 0.82      | N     |
| Gaspar colorectal adenoma | Bcl6    | MAPK4                         | 6.80×10⁻³ | 0.36      | *, #  |
| Rohrbeck all-Lung (cancer only) | Bcl6 | MAPK4                         | 2.50×10⁻³ | 0.45      | *     |
| Boersma Breast epithelial cancer | Bcl6 | MAPK7                         | 2.30×10⁻³ | 0.43      | *, #  |
| Grutzmann Pancreas all (no met) | ZBTB7A | MAPK27                        | 5.10×10⁻² | 0.6       | *, §  |
| Grutzmann Pancreas ductal adenocarcinoma | ZBTB7A | MAPK27                        | 1.20×10⁻² | 0.52      | A     |
| Zhai Cervix Cervical squamous | ZBTB7A | MAPK27                        | 1.30×10⁻² | 0.46      | *, #  |
| Zhai Cervix all           | ZBTB7A     | SCRIB                         | 3.40×10⁻² | 0.34      | A     |
| Collisson Pancreas all-adenocarcinoma | ZBTB7A | SRC                           | 3.10×10⁻² | 0.42      | *     |
| Gaspar colon all          | Bcl6        | YES1                          | 1.60×10⁻⁵ | 0.47      | A     |
| Gaspar colorectal adenoma | Bcl6        | YES1                          | 1.40×10⁻⁴ | 0.49      | *, #  |
| Gaspar colon normal       | Bcl6        | YES1                          | 3.40×10⁻² | 0.45      | N     |
| Rohrbeck all-Lung (cancer only) | Bcl6 | YES1                          | 9.60×10⁻³ | 0.4       | *     |
| **Negative correlations** |             |                               |         |           |       |
| Gaspar colon all          | Bcl6        | DLG2                          | 2.20×10⁻¹⁵ | −0.75     | A     |
| Gaspar colorectal adenoma | Bcl6        | DLG2                          | 7.40×10⁻¹³ | −0.79     | *, #  |
| Gaspar colon normal       | Bcl6        | DLG2                          | 8.40×10⁻⁴  | −0.66     | N     |
| Rohrbeck all-Lung (cancer only) | Bcl6 | DLG2                          | 2.00×10⁻²  | −0.36     | *     |
| Rohrbeck all-Lung (cancer only) | Bcl6 | LLGL1                         | 3.80×10⁻³  | −0.44     | *     |

A, all (normal and cancer); N, normal; * Bcl6/ZBTB7 high and correlated as expected with the test gene; # trend is stronger in cancer compared with normal tissue; § borderline significance with positive correlation.
et al., 2013; C.P., A.B., H.R., unpublished data). *RhoGEF2* or *Src64B* cooperation with *ab* showed similar properties (Fig. 4C). By contrast, *DRac1 RasV12* tumours showed strong cell-autonomous overgrowth and invasive properties, whereas *DRac1 ab* expressing cells did not overgrow relative to wild-type tissue, but instead the surrounding wild-type tissue was induced to overgrow (Fig. 4C).

The phenomenon of non-cell autonomous overgrowth observed in *DRac1 ab* mosaic eye-antennal discs (and to some extent in *ab* *RhoGEF2* and *ab Src64B* mosaic discs) is similar to the effect that “undead” cells (cells where apoptosis is initiated by activation of initiator caspases, but effector caspase activation is blocked – and thus cell death – by expression of the inhibitor, p35) have upon their surrounding wild-type neighbours (Martin et al., 2009; Morata et al., 2011; Perez-Garijo et al., 2009; Ryoo and Bergmann, 2012). This occurs by the release of Wingless (*Wg*) and Decapentaplegic (*Dpp*) and perhaps other morphogens from the undead cells, which promote compensatory proliferation in the surrounding wild-type cells. The similarity of these phenotypes suggests that *DRac1 ab* expressing cells might be in an “undead” state, and release Dpp and Wg, thereby inducing proliferative overgrowth of the surrounding wild-type cells. Alternatively, these cells might be deficient in mitochondrial function, which together with expression of a cell-survival factor, such as *RasV12*, results in non-cell autonomous overgrowth without evidence of caspase activation (Ohsawa et al., 2012). In this scenario, the mitochondrial dysfunction results in increased reactive oxygen species (ROS) that activate JNK signalling, which subsequently inactivates Hippo pathway signalling, leading to increased expression of the target genes Wingless and Unpaired (*Upd*) that activate Wg signalling and Jak/Stat signalling, respectively, in the neighbouring wild-type cells. However, since we observed TUNEL-positive cells in *DRac1 ab, RhoGEF2 ab* and *Src64B ab* expressing clones, it is more likely that the first of these mechanisms is responsible for the non-cell autonomous overgrowth, however this requires further investigation. Interestingly, in undead cells JNK activation is required for Dpp and Wg production and non-cell autonomous overgrowth (Morata et al., 2011; Perez-Garijo et al., 2009). Furthermore, strong activation of JNK signalling together with *RasV12* results in non-cell autonomous overgrowth (Uhlirova et al., 2005), although at presumably lower levels of JNK activation, cell autonomous overgrowth occurs (Brumby et al., 2011; Igaki et al., 2006; Uhlirova and Bohmann, 2006). Therefore it is possible that the different effects on non-cell autonomous versus autonomous cell overgrowth in *DRac1 ab* versus *RhoGEF2 ab* or *Src64B ab*-expressing cells might depend on the level of JNK activation. Nonetheless, at early stages, *ab*-driven *RhoGEF2*, *Src64B* or *DRac1* tumours were similar in inducing non-cell autonomous effects, but at later times the *RhoGEF2 ab* and *Src64B ab*-expressing cells showed more predominant autonomous cell overgrowth, whilst the DRac1 ab expressing cells did not, suggesting that there are likely to be molecular differences between *DRac1* and *RhoGEF2* or *Src64B* in their cooperative interactions with *ab* that impact on cell proliferation or survival of the tumour cells.

Our profiling of Ab targets and deregulated genes revealed that *dac, dan,eya* and *ct* eye-antennal differentiation genes were repressed, along with changes in expression of cell growth/proliferation and survival genes that would be expected to promote tumorigenic growth in cooperation with *scrib* loss-of-function (Turkel et al., 2013). *scrib* ab tumours showed downregulation of Dac, but the antennal cell fate expression domain of DII was not affected (Turkel et al., 2013). Similarly, *ab* expression with either of the cytoskeletal genes resulted in repression of Dac, however *Src64B ab* tumours additionally repressed DII, in contrast to *DRac1 ab, RhoGEF2 ab* and *scrib* ab tumours where DII was unaffected. This data suggests that *Src64B*
epithelial cancers, the correlations observed between elevated ZBTB7A expression and reduced expression of the Scribble polarity module gene (or high expression of cytoskeletal genes) might also indicate a requirement for glycolytic pathway activation for tumorigenesis. Further studies are clearly required to examine the cooperative effects of Bcl6 or ZBTB7A with deregulated cytoskeletal or cell polarity genes in human epithelial cell lines and mouse models in order to discern whether our findings in Drosophila are indeed conserved in mammalian systems.

Identifying cooperative interactions in cancer is likely to provide novel therapeutic approaches in combating the tumour. Indeed, recently a small molecule inhibitor targeting Bcl6 has been developed, and combining this with a Stat3 inhibitor resulted in enhanced cell killing in triple negative breast cancer cell lines (Walker et al., 2014). Since in Drosophila and human cells, Src upregulates Stat activity (Cordero et al., 2014; Frame, 2004; Kohlmaier et al., 2014; Read et al., 2004; Sotillos et al., 2013), tumours showing high Bcl6 and Src or Yes1 expression would be predicted to be sensitive to this combined therapeutic regime. Interestingly, a predominance of the significant correlations that were observed in the human epithelial cancer datasets with either Bcl6 or ZBTB7A involved upregulation of JNKK and JNK family genes. Since JNK signalling is central to many cooperative interactions examined by us and others (Brumby et al., 2011; Brumby and Richardson, 2003; Enomoto and Igaki, 2013; Igaki et al., 2006; Leong et al., 2009; Turlkel et al., 2013; Uhlirova and Bohmann, 2006), inhibiting the JNK pathway in addition to Bcl6 in Bcl6-driven cancers might also be a promising therapeutic approach to combat these cancers. In summary, our functional studies in Drosophila and bioinformatics analysis of human cancers has shown that cooperative tumorigenic interactions occur between BTB-ZF genes and cell polarity or cytoskeletal genes, and warrants further investigation to determine whether restoring normal expression of these genes or downstream pathways in human cancer cells can reduce tumorigenesis.

**Cooperation of BTB-ZF transcription factors with deregulated cytoskeletal or polarity genes in human cancer**

Our finding that there was a significant correlation between increased expression of human BTB-ZF oncogenic genes, Bcl6 or ZBTB7A, and downregulation of the cell polarity genes, Dlg2 and Lgl1, or homologs of JNKK (MAPK2K4, MAPK2K7), JNK (MAPK8, MAPK9, MAPK10), RhoGEF2 and DRac1 also upregulate JNK, downregulate the E-cadherin/b-catenin adhesion complex and repress Hippo signalling (Brumby and Richardson, 2003; Doggett et al., 2011; Igaki et al., 2006; Leong et al., 2009; Uhlirova and Bohmann, 2006). Furthermore, the Jak/Stat ligand, Upd3, is also upregulated in the scrib cells, where it drives tumour overgrowth, and is also required to activate Jak/Stat signalling in the wild-type neighbouring cells in cell competition (Bunker et al., 2015; Chen et al., 2012; Schroeder et al., 2013). RhoGEF2 and DRac1 also upregulate JNK signalling (Brumby et al., 2011; Khoo et al., 2013), and might also repress Hippo signalling to promote tissue growth, since regulators of actin cytoskeletal tension, such as activated Rok and Myosin II regulatory light chain, induce Yki target gene expression (Fernandez et al., 2011; Halder et al., 2012; Rauskob et al., 2014; Sansores-Garcia et al., 2011). However, in Drosophila it is unknown if RhoGEF2 or DRac1 affect Jak/Stat signalling. Since scrib loss-of-function and Src activation deregulate similar pathways, the precise mechanism by which Src64B cooperates with ab to block expression of DII in the developing eye-antennal disc remains to be determined.

**MATERIALS AND METHODS**

**Drosophila stocks**

The following Drosophila stocks were used: ey-FLP1, UAS-mCD8-GFP;; Tub-GAL4, FRT82B, Tub-GAL80 (Lee and Treisman, 2001); UAS-ab55 (III) (Cook et al., 2004); UAS-RhoGEF2 (II) (Padash Barmchi et al., 2005); UAS-Src64B (II) (R. Cagan, Mount Sinai School of Medicine, New York, USA); UAS-DRac1 (II) (Luo et al., 1994); scrib (III) (Bilder and Perrimon, 2000) and ey-GAL4 (Bloomington Stock Centre). FRT82B recombinant stocks were generated for all transgenic lines for mosaic analysis.

**Immunostaining**

Third-instar larval eye-antennal discs were dissected in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 30 min, and washed in PBS +0.1% Triton X-100 (PBT). Samples were blocked in 2% NGS in PBT with 1.5% saponin for 1 h in room temperature and then incubated in primary antibodies overnight at 4°C in 2% NGS in PBT. Samples were then washed two times in PBT for 30 min before addition of the secondary antibody. EdU and TUNEL labelling were performed as previously described (Turkel et al., 2013).

**Mosaic analysis**

Clonal analysis utilised MARCM (mosaic analysis with repressible cell marker) (Lee and Luo, 1999) with FRT828 and ey-FLP1 to induce clones and mCD8-GFP expression to mark mutant tissue. All fly crosses were carried out at 25°C and grown on standard fly media.
F-actin was detected with phallloidin–tetramethylrhodamine isothiocyanate (TRITC; Sigma, 0.3 μM, 1/1000) and DNA was detected using DAPI staining. Samples were mounted in 80% (v/v) glycerol/PBS.

Imaging
Images of fixed and mounted samples onto the glass slides were captured using BioRad, Olympus Fluoview FV1000 and Leica TCS SP5 confocal laser microscopes. Single optical sections were selected in Fluoview software before being processed in Adobe Photoshop to assemble into figures in Adobe Illustrator CS6.

Adult flies were frozen at −20°C before imaging in order to facilitate positioning them under the microscope. Images were captured on Lumenera Infinity 1 camera attached to Olympus SZX7 dissection microscope and processed using Adobe Photoshop CS3.

Quantification of clone volume
Volumetric clone analysis was performed using Velocity 3D Image Analysis Software (Perkin-Elmer). To determine the ratio of clonal tissue volume to total volume of the eye-antennal disc for each genotype and time point, GFP+ clonal tissue relative to total disc area (as marked by Phalloidin to visualize the cells) was measured from confocal Z sections encompassing the entire eye-antennal disc. The data for each genotype was compared using GraphPad Prism 6 using unpaired t-tests. Error bars represent s.e.m. and the significance was set at P<0.05.

Quantification of EdU and TUNEL staining
For TUNEL and EdU labelling, 6 to 10 discs for each genotype were analysed. TUNEL was quantified using Photoshop 5.1 Extended. EDU was quantified using a program designed by David Tapia dor, available at https://github.com/nogates/counting-semaphore.

Analysis of published datasets
Using Oncomine (Research Premium Edition), we identified 18 published gene expression data sets that contain epithelial cancer samples. Data was filtered down to the genes of interest and was downloaded for further analysis. Eleven of the 18 data sets that have at least 30 samples and contain at least three quarters of our query genes were analysed for correlation of expression levels between BCL6/ZBTB7A and each of the cancer-specific gene expression correlations. All analyses were done using the R software package.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
N.T., M.P., H.E.R. and A.M.B. conceived and designed the experiments. N.T., M.P. and C.P. performed the experiments. N.T., M.P., J.L., A.M.B. and H.E.R. analysed the data. N.T., M.P. and H.E.R. wrote the paper.

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