Notch and Mef2 synergize to promote proliferation and metastasis through JNK signal activation in *Drosophila*

SK Pallavi¹, Diana M Ho¹,
Chindo Hicks², Lucio Miele² and
Spyros Artavanis-Tsakonas^{1,*}

¹Department of Cell Biology, Harvard Medical School, Boston, MA, USA
and ²Cancer Institute at University of Mississippi Medical Center,
Jackson, MS, USA

Genetic analyses in *Drosophila* revealed a synergy between Notch and the pleiotropic transcription factor Mef2 (myocyte enhancer factor 2), which profoundly influences proliferation and metastasis. We show that these hyperproliferative and invasive *Drosophila* phenotypes are attributed to upregulation of *eiger*, a member of the tumour necrosis factor superfamily of ligands, and the consequent activation of Jun N-terminal kinase signalling, which in turn triggers the expression of the invasive marker MMP1. Expression studies in human breast tumour samples demonstrate correlation between Notch and Mef2 paralogues and support the notion that Notch–MEF2 synergy may be significant for modulating human mammary oncogenesis.

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Introduction

The Notch signalling pathway defines one of the fundamental cell signalling mechanisms in metazoans, and is broadly responsible for controlling cell fates by profoundly affecting proliferation, differentiation and apoptosis throughout development (Artavanis-Tsakonas *et al.*, 1999; Bray, 2006; Kopan and Ilagan, 2009; Artavanis-Tsakonas and Muskavitch, 2010). The Notch surface receptor interacts with membrane-bound ligands present on adjacent cells to trigger a proteolytic cascade that eventually cleaves the receptor and releases the intracellular domain (N^{act}) that subsequently translocates into the nucleus and participates in a transcriptional complex directing Notch-dependent transcription (Borggreve and Oswald, 2009). Development of both vertebrate and invertebrate animal systems has been shown to be very sensitive to the dosage of Notch signalling. Dysregulation of Notch signalling almost invariably leads to mutant phenotypes and other human diseases including cancer (Mohr, 1919; Aster and Pear, 2001; Gridley, 2003).

*Corresponding author. Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA.
Tel.: +1 617 432 7048; Fax: +1 617 432 7050;
E-mail: artavanis@cb.med.harvard.edu

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The genetic circuitry capable of modulating Notch activity has been reported to be very complex (Kankel *et al.*, 2007; Krejci and Bray, 2007), as is common with ancient conserved pathways, where regulatory mechanisms are numerous and diverse (Bray, 2006). Abnormal activation of the pathway has been shown to be oncogenic based on the observations that Notch behaves as an oncogene and activating mutations have been detected in almost half the cases analysed of T-cell acute lymphoblastic leukaemias (T-ALLs; Ellisen *et al.*, 1991; Weng *et al.*, 2004). Notch mutations in other tumours remain scarce or non-existent, yet there is a growing list of solid cancers, including breast, prostate, skin, brain, lung, colon and pancreas (Ranganathan *et al.*, 2011), where Notch activity has been positively correlated with oncogenic events.

We and others have shown that the activation of the Notch receptor can induce proliferation in a context-specific manner both in invertebrates and in vertebrates (Berry *et al.*, 1997; Go *et al.*, 1998; Baonza and Garcia-Bellido, 2000; Fre *et al.*, 2005). Our own studies in the mouse intestine and mammary glands (Kiaris *et al.*, 2004; Klinakis *et al.*, 2006; Fre *et al.*, 2009), however, lead to the conclusion that the activation of the Notch receptor *per se* is not necessarily oncogenic, but it is the synergy between Notch signals and other factors that drives proliferation, eventually leading to oncogenesis (Artavanis-Tsakonas and Muskavitch, 2010). These types of synergies may result in either the *de-novo* activation of new pathways or the enhancement of existing Notch target pathways, in both cases resulting in the acquisition of dramatic downstream functional changes.

We sought to identify genes that can synergize with activated forms of Notch to trigger proliferative events using genetic screens in *Drosophila* as our tool. Such synergies have been uncovered before in *Drosophila* (Moberg *et al.*, 2005; Ferres-Marco *et al.*, 2006; Vallejo *et al.*, 2011), suggesting the existence of several factors that can influence proliferation through synergistic or additive effects with Notch signals.

The screen we carried out resulted in the identification of Mef2 (myocyte enhancer factor 2) as a crucial synergistic partner of Notch in triggering massive proliferation and an invasive metastatic phenotype. *Mef2*, a transcription factor, was first identified as a regulator of muscle gene expression (Gossett *et al.*, 1989) that is required for myoblast differentiation (Bour *et al.*, 1995; Lilly *et al.*, 1995; Ranganayakulu *et al.*, 1995) but also has broad and pleiotropic effects in development and disease (Potthoff and Olson, 2007; Homminga *et al.*, 2011). Our mechanistic studies indicate that the synergy between Notch and Mef2 can be attributed to the activation of the Jun N-terminal kinase (JNK) signalling pathway. We furthermore show that this results in upregulation of the secreted ligand *eiger*, the orthologue of mammalian tumour necrosis factor (TNF) family cytokines. We examine the relevance of these results in human breast cancer samples and uncover a correlation

between Notch and Mef2 human paralogues, suggesting that this synergy is likely to be relevant to human malignancies.

Results

Notch and Mef2 synergy affects proliferation

The activation of the Notch receptor has been shown to induce proliferation in *Drosophila* (Go *et al*, 1998; Baonza and Garcia-Bellido, 2000) and in vertebrates (Fre *et al*, 2005; van Es *et al*, 2005). Constitutively activated Notch is oncogenic in several distinct contexts, often in combination with other factors (Radtke and Raj, 2003; Ranganathan *et al*, 2011). In order to identify these factors, we sought to carry out a genetic screen for modifiers of a ‘large eye’ phenotype induced by the overexpression of a constitutively active, ligand-independent form of the Notch receptor (N^{act}), that has been shown to be oncogenic in mammals (Kiaris *et al*, 2004).

Ectopic expression of N^{act} in the *Drosophila* eye, using the eye-specific driver *E1Gal4* (*eyGal4*), results in a large eye phenotype (Figure 1B) caused by elevated levels of cell proliferation as shown by an increase in EdU incorporation levels accompanied by slightly larger and distorted eye discs (Figure 1E). This proliferation increase is heterogeneous, resulting in spots of localized EdU incorporation within the undifferentiated (Elav-negative) cell compartment, both because of the non-homogeneous expression pattern of the *E1Gal4* driver and because of context specificity. The phenotype is dosage sensitive to Notch signal modulation and, as expected, could be enhanced or suppressed using gain- and loss-of-function mutations in Notch pathway elements and could therefore be used as the experimental parameter in a genetic screen for Notch modifiers. Using the Exelixis collection of insertional mutations, which covers ~50% of the *Drosophila* genome (Kankel *et al*, 2007), we identified two separate Gal4-driven mutations, d06622 and d03191, that strongly enhanced the large eye phenotype caused by N^{act} (Figure 1C).

Mapping of the insertion (Thibault *et al*, 2004) revealed that these mutations represent two separate gain-of-function alleles of *Mef2*, a pleiotropic, essential gene that belongs to the evolutionarily conserved MADS family of transcription factors implicated in the development of diverse tissues (Potthoff and Olson, 2007). *Drosophila* possesses a single *Mef2* gene that codes for an ~57 kilodalton protein (Nguyen *et al*, 2002), whereas vertebrates harbour four genes (*Mef2* A, B, C, and D) (Potthoff and Olson, 2007). Western blot and immunofluorescence analyses of both alleles revealed elevated expression levels of the *Mef2* protein compared with wild type, proving the nature of the mutations (Supplementary Figure S1).

Neither of the Exelixis *Mef2* alleles on their own affected the adult eye morphology and the corresponding eye discs appeared wild type (Figure 1F). In contrast, coexpression of a single copy of either of these two *Mef2* alleles with N^{act} resulted in massively overgrown discs, showing excessive EdU incorporation (Figure 1G). Expressing an *Mef2* transgene caused an even stronger synergy with N^{act} (Supplementary Figure S1). Costaining with the neuronal differentiation marker *Elav* revealed that the hyperproliferative cell compartment was restricted to the anterior of the morphogenetic

furrow harbouring the undifferentiated cells of the eye disc (Figure 1G).

To examine whether the observed synergy is confined to the eye or is a more general phenomenon, we extended our analysis to the developing wing and coexpressed N^{act} and *Mef2* under two different wing-specific drivers, *vgGal4* and *MS1096Gal4*. Compared with discs expressing the two genes individually, coexpression resulted, just as in the eye, in massively overgrown discs (Supplementary Figure S1), indicating that the synergy is not tissue specific.

Emphasizing the synergistic nature of the phenotype, only the coexpression of N^{act} and *Mef2*, not either gene alone, in somatic clones of the eye discs induced the expression of the Notch target *Wingless* outside of its normal expression realm (Figure 1H–J), that is, the lateral margins of the eye discs (Treisman and Rubin, 1995), releasing Notch signalling from its normal context-dependent restrictions. As in the eye, coexpression of N^{act} and *Mef2* resulted in the synergistic activation of *wingless* in the wing, outside of its normal expression domain of the wing margin (Couso *et al*, 1994; Supplementary Figure S1). The activation of ectopic *wingless* by N^{act} and *Mef2* demonstrates that these two genes together but not alone can synergistically affect downstream gene expression.

We thus conclude that it is the synergy between N^{act} and *Mef2* that is responsible for inducing hyperproliferation and the accompanying phenotypes.

Notch and Mef2 synergy induces metastatic behaviour and MMP1 expression

In addition to hyperproliferation, coexpression of N^{act} and *Mef2* in the eye consistently, albeit at low penetrance (~5%), causes ectopic eyes in the thorax or abdomen (Figure 2A). Similar phenotypes in *Drosophila* have been previously reported and associated with invasive and metastatic behaviour (Ferres-Marco *et al*, 2006; Palomero *et al*, 2007).

Matrix metalloproteinases (MMPs) have been shown to degrade basement membrane and are thus required for the metastatic behaviour of cells in both mammals (McDonnell *et al*, 1991; Wang *et al*, 2010) and *Drosophila* (Uhlirva and Bohmann, 2006). Therefore, we used MMP1 as a molecular marker to further characterize the N^{act} and *Mef2* invasive phenotype.

Wild-type wing discs show endogenous MMP1 in the trachea and a small region of the notum, consistent with previously published data (Figure 2B) (Page-McCaw *et al*, 2003). Expression of N^{act} using the wing pouch-specific driver *MS1096Gal4* only occasionally causes a detectable upregulation of MMP1; however, MMP1 expression is markedly and consistently increased in *Mef2* expressing wing discs (Figure 2C and D). We note that, even though *Mef2* is ectopically expressed in the entire pouch region, MMP1 upregulation is seen only near the dorsal/ventral (D/V) boundary (as marked by the *vg-LacZ* reporter; Supplementary Figure S2A), which is also the defined area of active endogenous Notch signalling (Neumann and Cohen, 1998), suggesting that *Mef2*-dependent MMP1 expression may require Notch signals.

This notion was corroborated by the fact that when we inhibit endogenous Notch activity using an RNAi against Notch in the D/V boundary of discs expressing *Mef2*, we detected a dramatic downregulation of the ectopic MMP1

Figure 1 Synergy between Notch and Mef2 in the eye. (A) Wild-type fly eye. (B) Activated Notch (N^{act}) in the eye results in a large eye when driven by the eye-specific *E1Gal4* driver. (C) Exelixis Mef2 fly stock ($d06622$) synergizes with N^{act} in the eye to cause massively overgrown eyes. (D–G) EdU incorporation assay in eye-antennal discs expressing various constructs under *E1Gal4*. Discs are labelled with EdU (red) to mark dividing cells and stained for anti Elav (green) to mark differentiated cells. (D) Wild-type control; # marks antennal disc (in which *E1Gal4* is not active). (E) N^{act} alone causes an increase in EdU levels (*) and (F) Mef2 ($d06622$) alone does not cause any change in EdU levels. On the other hand, (G) N^{act} together with Mef2 ($d06622$) causes an enormous increase in both EdU labelling and disc size. Note that EdU-positive cells appear to be restricted to the Elav-negative undifferentiated cells. (H–J) Notch and Mef2 synergize to induce expression of the Notch target gene wingless in the eye disc. Clones expressing N^{act} and/or Mef2 were generated (see Materials and methods). Eye-antennal discs containing GFP-labelled clones were stained with wingless (wg, red). Note that neither N^{act} (H) nor Mef2 (I) alone could induce wg in the eye, but together they turned on ectopic wg in all clones (J).

expression (Figure 2G). When N^{act} and Mef2 are coexpressed, MMP1 is highly upregulated throughout the entire wing disc (Figure 2E; Supplementary Figure S2B). To ensure that these observations are not specific to the MS1096Gal4 driver, we repeated this analysis using another wing driver, *vgGal4*, and obtained similar results.

To extend this analysis we generated somatic clones over-expressing N^{act} and Mef2 and, consistent with the above observations, ectopic expression of N^{act} alone did not induce MMP1 in clones (Figure 2H and H'). Mef2 alone induced some punctate expression of MMP1 only in clones that were located near the D/V boundary but not in clones located away from the boundary (Figure 2I and I'). On the other hand, coexpression of N^{act} and Mef2 resulted in high levels of MMP1 in every single clone regardless of its position relative to the D–V boundary (Figure 2J and J'). We furthermore find that the basement membrane immediately surrounding N^{act} + Mef2 clones is disrupted as indicated by gaps in the laminin expression pattern (Supplementary Figure S2D and D'). Thus, the synergy between N^{act} and Mef2 not only causes

hyperproliferation but also induces MMP1 expression and basement membrane degradation.

The ectopic eye phenotype and MMP1 upregulation seen in cells coexpressing N^{act} and Mef2 all point to the possibility that these cells have acquired invasive characteristics. To test this hypothesis directly, we asked whether cells confined to a specific cellular compartment could be induced to cross the boundary to another compartment upon coexpression of both N^{act} and Mef2.

For this, we used the decapentaplegic-Gal4 (*dppGal4*) driver, which is expressed in the anterior compartment of the A/P boundary of the wing imaginal disc (Raftery *et al*, 1991); since Dpp represses *engrailed* (*en*), cells expressing *dppGal4* and *En* are mutually exclusive, creating, in normal wing discs, a clearly defined and contiguous A/P boundary (Sanicola *et al*, 1995). We used *dppGal4* to express N^{act} and/or Mef2, along with GFP to mark *dppGal4*-expressing cells, and asked whether or not GFP-labelled cells could migrate away from the boundary into the *En* expression domain. In clones expressing N^{act} alone we never saw GFP-positive cells

Figure 2 Notch and Mef2 synergize to induce MMP1 expression and invasiveness. (A) An adult fly coexpressing N^{act} and the Exelixis Mef2 stock d06622 under E1Gal4 displays an ectopic eye in the abdomen (arrow). (B–G) Wing discs expressing various UAS constructs (as indicated) under *MS1096*Gal4 were stained for MMP1 (green). Note that Mef2 alone (D) induces MMP1 expression only in the D/V boundary, which is reduced by RNAi-mediated downregulation of endogenous Notch (G). In contrast, N^{act} and Mef2 together (E) strongly induce MMP1 throughout the entire wing disc as compared with wild type (B) or N^{act} alone (C). (H–J) GFP-positive clones (green) expressing N^{act} and/or Mef2 were generated (see Materials and methods) and stained for MMP1 (red). (H, H') N^{act} does not induce MMP1 in clones, (I, I') Mef2 induces some MMP1 only in clones near the D/V boundary (arrowheads; D/V boundary is marked by a dotted line) but not in clones that are further away (arrows, clone boundaries marked by dashed yellow line), and (J, J') N^{act} and Mef2 together induce MMP1 in all clones regardless of their location. (K–N) Wing discs expressing various UAS constructs (as indicated) along with UASGFP under *dpp*Gal4 were stained for engrailed (*en*, red), which is mutually exclusive of the *dpp*Gal4 domain. The A/P boundary is marked with a dotted line. Low magnification images showing the topology of the wing disc are shown in K, L, M, and N. (K–K'') Both control discs and discs expressing N^{act} alone show restricted expression of *dpp*Gal4/UASGFP along the A/P boundary, although in the N^{act} discs we see overgrowth of the *dpp*Gal4 region, but all growth is into the anterior compartment of the discs and a distinct A/P boundary is maintained. (M–M'') Mef2 expression alone results in single cells marked by GFP (arrow in M'') invading the posterior compartment of the wing disc counterstained by *en*. (N–N'') Coexpression of N^{act} and Mef2 not only causes very strong *dpp*Gal4/UASGFP staining along the A/P boundary but also results in groups of GFP-positive cells (arrows) invading the posterior compartment, characteristic of invasive metastatic tissue. These groups of cells do not express *en* (circle in N''), indicative of their anterior nature. All wing discs are oriented with dorsal to the top and posterior to the right.

mixing with the En cells (Figure 2L and L''). In contrast, single GFP-positive cells were seen some distance away from the A/P boundary among the En-positive cells upon overexpression of Mef2 alone (Figure 2M and M''). These single cells may be apoptotic given their morphology, especially given that Mef2 causes an increase in caspase-3 cleavage (see data below in Figure 6). Most strikingly, when N^{act} and Mef2 were coexpressed, we observed larger clusters of cells invading the En domain (Figure 2N and N''). Notably, the GFP-positive cells in these clusters did not themselves express *en*, indicating that they are indeed cells that have migrated out of the *dpp*Gal4 domain.

This invasive behaviour of N^{act} and Mef2 coexpressing cells was further explored using a larval metastasis assay established by Pagliarini and Xu (2003), in which gene

expression is driven in eye discs by *ey*Flp; clusters of cells (which may be as small as single cells) marked with GFP are then monitored for migration into the larval body. Consistent with the *dpp*Gal4 results, N^{act} alone did not induce any migration of GFP-labelled cells while Mef2 expression did result in a few migrating cells (average no. of clusters = 4). These primarily appeared to be single cells rather than larger clusters. This Mef2 phenotype was significantly enhanced when N^{act} was coexpressed, such that both the size and number of ectopic GFP-positive cell clusters was markedly increased (average no. of clusters = 6) (Supplementary Figure S3).

Live imaging of GFP-labelled somatic clones coexpressing N^{act} and Mef2 also revealed that individual GFP-positive cells could be seen migrating away from the main cluster of cells

within a 45-min timeframe (Supplementary Movie S1). In contrast, the cells in clones expressing only N^{act} remained as a static cluster (Supplementary Movie S2). Clones expressing Mef2 alone do not show any movement, although they do send out transient processes (Supplementary Movie S3). We note that Mef2 clones are smaller than N^{act} + Mef2 clones because the synergy between N^{act} and Mef2 results in increased proliferation and hence a larger clone; therefore, we cannot exclude the possibility that the lack of migrating cells seen in Mef2 clones could be due to decreased cell numbers, a notion that is supported by the fact that we do indeed see a few migrating Mef2 cells in the dppGal4 and larval metastasis assays.

Notch–Mef2 synergy affects epithelial integrity

A noteworthy aspect of clones coexpressing N^{act} and Mef2 is that they display a distinctive morphology. These clones are rounded, sometimes even forming hollow ball-shaped structures, and often appear to be protruding from the flat epithelial cell layer of the disc (Figure 3C and C'), consistent with the notion that N^{act} and Mef2 coexpression causes disruption of epithelial integrity, leading to metastatic cellular behaviour. We were thus prompted to examine key molecules previously linked with loss of epithelial integrity (Rodahl *et al*, 2009), such as actin and β -integrin.

Clones expressing N^{act} alone showed no detectable change in either actin or β -integrin expression (Figure 3A–A' and D–D'). Mef2 expression caused an upregulation of actin and β -integrin levels, which was clearly further enhanced by coexpressing N^{act} (Figure 3B–B', E–E' and C–C', F–F'). Discs large (Dlg), which normally localizes to basolateral junctions, is also upregulated and mislocalized throughout N^{act} + Mef2 clones, including at the apical surface (Supplementary Figure S2E and E'), raising the possibility of a loss of normal apicobasal polarity. Notably, the highest levels of actin and β -integrin were observed at the clone borders raising the possibility that these cells may have altered cell adhesion properties, which could promote formation of cellular

ensembles that can thus escape the epithelial integrity of the surrounding wild-type cells.

Mef2 is linked to the JNK signalling pathway

It has been previously demonstrated that the JNK signal activation due to mutations in *scribbled*, a *Drosophila* tumour suppressor gene, causes an upregulation of MMP1, resulting in invasive cellular behaviour (Uhlirva and Bohmann, 2006). Furthermore, upregulation of JNK signalling has been linked to the control of both epithelial integrity and proliferation. We were thus led to examine whether the mechanism underlying the invasive, hyperproliferative behaviour of N^{act} and Mef2 cells is related to JNK signalling.

To monitor JNK signal activation, we used the transgenic reporter, *puckered-lacZ* (*puc-LacZ*) (Martín-Blanco *et al*, 1998). *Puckered* is a transcriptional target as well as a feedback inhibitor of JNK. Activating N^{act} alone in the D/V boundary cells using *vgGal4* caused a slight upregulation of *puc-LacZ* (Figure 4B). In contrast, Mef2 alone led to a clear upregulation of *puc-LacZ*, which was significantly amplified upon coexpression of N^{act} (Figure 4C and D; Supplementary Figure S2C). Clonal analysis also showed the same result (Supplementary Figure S4).

Blocking the JNK pathway rescues both invasiveness and overgrowth

If JNK signalling is the crucial event downstream of the N^{act} and Mef2 synergy, then one might expect that blocking JNK signals should rescue the synergistic phenotype. We thus blocked the JNK pathway using a dominant-negative allele of the *Drosophila* JNK gene *Basket*, *Basket^{DN}* (*Bsk^{DN}*) (Adachi-Yamada *et al*, 1999). Strikingly, expression of *Bsk^{DN}*, along with N^{act} and Mef2, suppressed the overproliferation phenotype, MMP1 upregulation, and *puc-LacZ* expression in wing discs (Figure 4F–M). *Basket^{DN}* alone does not have any appreciable effect on disc size or MMP1 expression (data not shown). It therefore appears that the N^{act} and Mef2 synergistic phenotype is dependent upon activation of the JNK pathway.

Figure 3 Notch and Mef2 synergize to disrupt epithelial integrity. (A–F') Clones expressing N^{act} and/or Mef2 were generated using hsflp; Actin5C > y^+ > Gal4; UASGFP (10', 37°C heat-shock 24–48 h (A–C') or 48–72 h (D–F') AEL) and stained with phalloidin (red, A–C'), or anti- β -integrin (red, D–F'). Coexpression of N^{act} and Mef2 upregulates the expression of the epithelial markers actin (C–C') and β -integrin (F–F') and appears to cause changes in cell adhesion properties. Note the characteristic nature of the N^{act} and Mef2 clones, which protrude out of the wing disc membrane as a rounded and well-defined group of cells (C, arrow). All wing discs are oriented with dorsal to the top and posterior to the right.

Figure 4 Blocking the JNK signalling pathway with Bsk^{DN} results in rescue of Notch + Mef2 synergy. (A–D) Wing discs expressing various UAS constructs (as indicated) under *vgGal4* and stained for *puc-LacZ* (green), a marker for JNK activity. Note that Mef2 induces *puc-LacZ* expression in the D/V boundary (C), which is enhanced many-fold when N^{act} is coexpressed (D). (E–I) DIC images of *vgGal4*-driven UAS constructs (genotypes as indicated) showing the varied size differences. Note that blocking the JNK pathway using Bsk^{DN} (I) partially rescues the N^{act} and Mef2-induced gigantic disc phenotype, and also reduces the expression of MMP1 (J, K; red) and *puc-LacZ* (L, M; green) to near normal levels. All wing discs are oriented with dorsal to the top and posterior to the right.

Notch and Mef2 synergy controls the expression of a JNK pathway-activating ligand

Given that the N^{act} and Mef2 synergistic effects depend on JNK signals, we sought a deeper mechanistic insight into this relationship.

The only known ligand that activates the JNK pathway in *Drosophila* is *eiger* (*egr*), a member of the TNF superfamily (Igaki *et al*, 2002a, b; Moreno *et al*, 2002). Overexpression of *Egr* along with N^{act} in wing discs resulted in a large disc phenotype very similar to that seen by coexpressing N^{act} and Mef2. MMP1 was also synergistically upregulated in these discs (Supplementary Figure S5), suggesting that the regulation of the JNK pathway could be at the level of the ligand. To investigate this, we performed immunofluorescence for *Egr* in clones overexpressing N^{act} and/or Mef2. Wing discs with

control LacZ-expressing clones show only endogenous *Egr* expression in the notum region of the disc, consistent with previously published data (Figure 5A–A''; Igaki *et al*, 2002a, b). Clones overexpressing N^{act} or Mef2 alone in the wing disc did not show any notable ectopic expression of *Egr*; however, every clone coexpressing N^{act} and Mef2 displayed upregulated levels of *Egr* (Figure 5B–D'').

To examine the role of *Egr* induction downstream of N^{act} and Mef2, we asked whether downregulating *Egr* could rescue the synergistic phenotypes. Loss of both copies of *egr* (using the loss-of-function allele *egr¹*) in a N^{act} and Mef2 overexpression background resulted in a suppression of the synergistic N^{act}/Mef2 overgrowth phenotype as assessed by the size of the wing disc (Figure 5H and I). There was a visually obvious decrease (in all larvae tested; *n* > 40) in the

Figure 5 Egr, a TNF ligand, is required downstream of N^{act} and Mef2 signals. (A–D'') Clones expressing N^{act} and/or Mef2 were generated (see Materials and methods) and stained for Egr (green). Clones are marked by lacZ (red: anti-β-gal). Normal expression of Egr is seen in the notum (*) in each genotype. Note the synergistic upregulation of Egr expression (green) by N^{act} and Mef2 together (D–D'') as compared with either alone (B–B'' and C–C''). Only along the D/V boundary do a few clones that express Mef2 alone display slight upregulation of Egr (arrows in C'). (E–I) Rescue of the N^{act} and Mef2-induced giant disc phenotype in *egr¹/egr¹* background. Expression of Mef2 (F–G) or N^{act} and Mef2 together (H–I) in the *egr¹/egr¹* background rescues MMP1 to near normal levels and partially rescues the N^{act} + Mef2-induced giant disc phenotype. All wing discs are oriented with dorsal to the bottom and posterior to the left. (J) Gel shifts performed using a 200-bp fragment of the *egr* promoter bearing Su(H) and MEF2-binding sites and a control fragment with the sites mutated (see Materials and methods). Whole protein extract from wild-type and N^{act} + Mef2 overexpressing wing discs show gel shifts using the wild-type fragment that could be competed using cold probe. A mutant version of the same fragment does not show significant gel shifts, and therefore no competition. (K–M) Supershifts using antibodies against N^{act} (K) and Mef2 (L, M), using 30 mer complementary oligonucleotides carrying the Su(H) or Mef2-binding sites from the *egr* promoter. (K) The shifted band using protein extract from w1118 wing discs was supershifted by N^{act} antibody but not by an antibody against cut. (L) Similarly, a supershift is seen by addition of Mef2 antibody. However, since the supershift effect with Mef2 antibody is weak with protein extract from w1118, we repeated this experiment using extract from N^{act} + Mef2 overexpressing wing discs, which have higher levels of protein and show a much clearer supershift (*M, also see Supplementary Figure S5).

levels of both MMP1 (Figure 5I) and *puc-LacZ* (data not shown), indicating that the N^{act} and Mef2 synergy depends on the presence of *egr*. In addition, the ectopic levels of MMP1 associated with Mef2 expression were also suppressed (Figure 5F and G). We thus conclude that *egr* is essential for the synergistic overgrowth phenotype induced by N^{act} and Mef2.

Examination of the DNA sequences upstream of the initiation site of *egr* shows binding sites for Mef2 and the transcriptional effector of Notch, Su(H) (Suppressor of Hairless). We were thus prompted to explore the possibility that the effect we observe on *eiger* may be mechanistically explained by a direct Notch–Mef2 synergy at the transcriptional level. A 200-bp fragment (–668 to –467) containing the putative binding sites from the *eiger* promoter region was examined for Mef2 and N/Su(H) binding using EMSA (electromobility shift assay). We observed a shift of the 200-bp band upon addition of protein lysate from both wild-type wing discs, which contains endogenous Notch and Mef2, and lysate from wing discs overexpressing N^{act} and Mef2, while the equivalent fragment in which the Mef2 and Su(H) binding sites are mutated is not shifted by the same extracts (Figure 5J). To corroborate the identity of proteins associated with the observed shift, small 30-mer oligos containing the Su(H) binding site or the Mef2 sites were subjected to supershift assays using N^{act} and Mef2 antibodies. These oligos show clear supershifts upon addition of their respective antibodies (Figure 5K–M; Supplementary Figure S6), consistent with the notion that the shift we observe on the 200-bp fragment is due to direct binding of N/Su(H) and Mef2.

To show that the binding sites are functionally significant, we cloned the same 200 bp *eiger* promoter element used in EMSA upstream of a luciferase reporter gene. Transient transfection of N^{act} and Mef2 together into S2R+ cells causes a 2.0-fold increase in reporter activity when compared with N^{act} or Mef2 alone (Supplementary Figure S6). We therefore conclude that the binding of Su(H) and Mef2 to the *eiger* promoter synergistically upregulates *eiger* expression, but we do note that testing only the 200-bp fragment may not reflect accurately the *in-vivo* situation.

Inhibition of the JNK pathway by *Diap1* rescues the N^{act} + Mef2 synergy

JNK signalling regulates various physiological processes by controlling not only proliferation but also apoptosis (Ryoo *et al*, 2004). Overexpression of N^{act} alone in the wing disc does not cause appreciable cell death as assessed by staining for cleaved caspase-3 but does, as expected, result in the upregulation of Wg, a known downstream target of Notch activity (Figure 6B–B''). Mef2 alone stimulates significant caspase-3 cleavage and a slight upregulation of Wg (Figure 6C–C''). Overexpression of N^{act} and Mef2 together is associated with a strong synergistic increase in Wg expression, but in this case we observe only a modest, apparently non-synergistic, induction of cleaved caspase-3 (Figure 6D–D'').

It has been previously shown that the *Egr* overexpression phenotype in the eye can be rescued by *Drosophila* inhibitor of apoptosis protein 1 (DIAP1) but not by the caspase inhibitor P-35 (Igaki *et al*, 2002a). Coexpression of DIAP1 together with N^{act} and Mef2 suppressed the overgrowth phenotype as assessed by both disc size and disc

morphology and strongly reduced the domain of Wg expression to a level near that seen when N^{act} is expressed alone (Figure 6E–E''). Analogous P-35 expression could rescue neither disc size nor Wg activation associated with the N^{act} and Mef2 synergy (Figure 6F–F''). DIAP1 has been reported to suppress caspase-independent cell death (Igaki *et al*, 2002b), which may account for our observation that we do not observe synergistic effects on cleaved caspase-3. Taken together, our results support a model in which N^{act} and Mef2 activate JNK signalling to cause hyperproliferation and invasiveness in an *egr*-dependent manner (Figure 7).

Correlation between Notch and Mef2 in human breast tumours

The hyperproliferation and metastasis phenotype associated with the N^{act} and Mef2 synergy seen in *Drosophila* prompted us to examine the possible relevance of these findings in human breast cancer, especially in view of the fact that dysregulation of Notch receptors has been implicated in breast cancer (Pannuti *et al*, 2010). We thus investigated the link between the four Notch (NOTCH1–4) receptors and the four human MEF2 (MEF2A–D) genes using publicly available gene expression data derived from 484 breast cancer patients (Wang *et al*, 2005; Desmedt *et al*, 2007). Since ER+ and ER– are different disease entities, we therefore analysed correlations between NOTCH and MEF2 within each disease entity separately. For ER+ ($N=343$) we found significant ($P<0.0001$) correlations between NOTCH2 and MEF2A, $r=0.23$; NOTCH3 and MEF2D, $r=0.23$; NOTCH3 and MEF2A, $r=0.22$; NOTCH1 and MEF2B, $r=0.22$; NOTCH1 and MEF2A, $r=0.21$; NOTCH4 and MEF2A, $r=0.31$. Within ER– patients ($N=141$), significant ($P<0.0001$) correlations were found between NOTCH2 and MEF2A, $r=0.32$; NOTCH3 and MEF2D, $r=0.21$; NOTCH3 and MEF2C, $r=0.22$; NOTCH3 and MEF2A, $r=0.22$; NOTCH1 and MEF2A, $r=0.23$; NOTCH4 and MEF2A, $r=0.27$. These results suggest a moderate association between the NOTCH and MEF2 genes in breast cancers.

We focused on ER– disease, where expression of NOTCH1 has been shown to be highest (Haughian *et al*, 2012). Within ER– disease, we identified a striking difference between tumours that did and did not recur. NOTCH1, the orthologue of *Drosophila* Notch, shows a statistically significant ($P<0.0001$) positive correlation with all four MEF2 paralogues in tumours that recurred ($r=0.41$ with MEF2A, 0.39 with MEF2B, 0.29 with MEF2C and 0.27 with MEF2D, respectively). In tumours that did not recur, NOTCH1 shows a significant negative correlation with MEF2C (-0.23) and no correlation at all with the other three paralogues. This suggests that coexpression of NOTCH1 and one or more MEF2 paralogues may identify ER– tumours that are likely to relapse. We then performed Kaplan–Meier survival analysis within the subset of ER– tumours that did relapse (Supplementary Figure S7). Within this subset, NOTCH1 significantly associates with poor survival, while MEF2 paralogues by themselves do not (Supplementary Figure S7). This is consistent with the hypothesis that one or more MEF2 paralogues act as NOTCH1 cofactors in ER– tumours.

Breast metastatic lesions are seldom resected surgically. Therefore, an analysis of protein expression comparable to the gene expression analysis we performed would be very difficult. However, we generated a tissue microarray that

Figure 6 N^{act} + Mef2-induced hyperproliferative phenotype is rescued by DIAP1. (A–A'') Wild-type wing disc and (B–F'') Wing discs expressing various UAS constructs (as indicated) under *vgGal4* were stained for cleaved caspase-3 (cc3, green), wingless (wg, red), and DAPI (blue). (B–B'') Overexpression of N^{act} turns on wg (a Notch target) and shows only a small amount of cc3. (C–C'') Mef2 overexpression results in increased cc3 in the *vgGal4* expression domain and weak upregulation of wg expression in the mesonotum region (*). (D–D'') Coexpression of N^{act} and Mef2, which results in massively overgrown discs, induces only modest levels of cc3 (D) coupled with excessive wg upregulation (D'). Coexpression of DIAP1 (E–E'') rescues both disc size and wg expression induced by N^{act} + Mef2, whereas coexpression of P-35 (F–F'') has no effect. All wing discs are oriented with dorsal to the top and posterior to the right.

includes 48 metastatic lesions of several types of epithelial malignancies and stained it with Notch1 and pan-MEF2 antibodies. Several types of metastatic cancers, including breast cancer that had metastasized to the lymph node, showed coexpression of Notch1 and Mef2 (Supplementary Figure S8). Again, we identified a statistically significant correlation ($r = 0.43$ across 48 samples; $P < 0.0028$) between expression of Notch1 and MEF2.

In conclusion, while our studies of breast cancer samples are currently only correlative and cannot prove the functional relevance of Notch–MEF2 synergy in human tumours, they parallel the *Drosophila* data, supporting the notion that Notch–MEF2 interactions may be significant for human mammary oncogenesis.

Discussion

We undertook a genetic modifier screen in *Drosophila* and identified a number of genetic modifiers of Notch signals that affect proliferation. Further examination of one of these modifiers, Mef2, established that its synergy with Notch signals directly triggers expression of the *Drosophila* JNK

pathway ligand *eiger*, consequently activating JNK signalling that profoundly influences proliferation and metastatic behaviour. It might perhaps be worth noting that metastatic behaviour in *Drosophila* may not be completely equivalent to mammalian metastasis, notwithstanding the fact that they share molecular signatures, for example, MMP activation.

Cancer is characterized by the deregulation of the balance between differentiation, proliferation and apoptosis; thus, it is not surprising that the Notch signalling pathway, which plays a central role in all these developmental events, is increasingly implicated in oncogenic events. The rationale of our study is based on the fact that synergy between Notch and other genes is key in understanding how Notch signals contribute to oncogenesis. It remains a remarkable fact that while activating mutations in the Notch receptor have been associated with >50% of T-ALLs, a search for mutations in other cancers, despite a few suggestive reports (Ranganathan *et al*, 2011), remains essentially unfruitful. Yet, correlative studies have linked Notch activity with a broad spectrum of human cancers (Lee *et al*, 2007; Chadwick *et al*, 2009) and our own work in mice (Kiaris *et al*, 2004; Fre *et al*, 2009) suggests that while Notch activation promotes proliferation,

Figure 7 Model of Notch–Mef2 synergistic activity. The Notch–Mef2 synergy acts upon JNK signalling and downstream events. Notch and Mef2 cooperate to directly turn on the JNK pathway ligand *eiger* at the transcriptional level, which results in activation of JNK signalling. This in turn results in increased cellular proliferation as well as the formation of metastatic groups of cells that can migrate away from the original source.

it is the synergy between Notch and other factors that eventually leads to cancer. Similar synergies have been identified before (Moberg *et al*, 2005; Ferres-Marco *et al*, 2006; Vallejo *et al*, 2011) but the extraordinary complexity of the gene circuitry that modulates the Notch pathway (Hurlbut *et al*, 2009) suggests that more such relationships will be uncovered as exemplified by our discovery of Mef2 as a Notch synergistic partner affecting proliferation.

The transcription factor Mef2 plays an essential role in myogenic differentiation, but several studies have also shown a broad pleiotropic role of Mef2 (Naya and Olson, 1999; Potthoff and Olson, 2007). Mef2 can integrate signals from several signalling cascades through chromatin remodelling factors and other transcriptional regulators to control differentiation events. Our study extends the functionality of Mef2 by uncovering the profound effect it can have on proliferation and metastatic cell migration in synergy with Notch signals.

We are not the first to link Mef2 with Notch. They have been linked before in the context of myogenesis both in *Drosophila* and in vertebrates. A ChIP-on-chip analysis of Mef2 target regions identified several Notch pathway components as potential Mef2 targets during *Drosophila* myogenesis (Sandmann *et al*, 2006). In human myoblasts, Mef2C was suggested to bind directly to the intracellular domain of Notch via the ankyrin repeat region, suppressing Mef2C-induced myogenic differentiation (Wilson-Rawls *et al*, 1999). Mef2 has also been reported to interact with the Notch coactivator MAML1 and suppress differentiation (Shen *et al*, 2006).

While upregulation of Mef2 alone does not show overt proliferation effects, our analyses demonstrate that *in vivo* it can activate MMP1. Even though we ectopically expressed Mef2 in the whole wing pouch, MMP1 expression was confined around the D/V boundary, where endogenous Notch signals are active. This effect of Mef2 overexpression depends

on Notch signals, a notion corroborated by the fact that inhibiting Notch activity by RNAi reverses the effects of Mef2 on MMP1.

The polarity gene *scribble* cooperates with Ras signalling to upregulate the JNK pathway, promoting invasiveness and hyperplasticity (Brumby and Richardson, 2003). However, the synergy seen in our study appears to be *scribble* independent. The fact that both the *scribbled*/Ras and the Notch/Mef2 metastatic pathways converge at the level of JNK signal activation suggests that JNK is a crucial regulator of oncogenic behaviour, which is controlled by inputs from multiple signals. Even though there is little evidence that *twist* activates JNK signalling, it is a crucial regulator of epithelial-to-mesenchymal transition and metastasis (Yang *et al*, 2006) and has also been independently linked to both Mef2 and Notch in myogenesis (Anant *et al*, 1998; Cripps *et al*, 1998). However, we note that the Notch–Mef2 synergy seems to be independent of *twist*, as Twist cannot replace Mef2 in the synergistic relationship (Supplementary Figure S9).

Numerous reports link JNK signalling to normal developmental events requiring cell movement and to metastatic phenomena both in *Drosophila* (Brumby and Richardson, 2003; Pastor-Pareja *et al*, 2004) and in vertebrates (Xia and Karin, 2004). JNK signals seem to be crucial for controlling gene activities involved in epithelial integrity and our observations from *Drosophila* suggest that the N^{act} and Mef2 synergy may be important in JNK-linked carcinogenesis. A role for Notch in controlling JNK signals has been reported previously (Zecchini *et al*, 1999; Kim *et al*, 2005). While the studies we carried out using breast cancer samples can only be correlative now, our observations suggest that metastatic breast tumours harbour higher levels of Notch and Mef2 paralogue pairs, consistent with our observations in *Drosophila*.

Although the majority of studies on Mef2 are focused on muscle development/differentiation, some intriguing links

between Mef2C and leukaemias are noteworthy. MEF2C and Sox4 synergize to cause myeloid leukaemia in mice (Du *et al*, 2005). Analysis of T-ALL patient samples revealed increased levels of Mef2C; however, Mef2C alone could not cause cellular transformation of NIH3T3 cells, but it could do so in the presence of RAS or *myc* (Homminga *et al*, 2011). Given the role of Notch in T-ALL it will be important to examine how activated Notch mutations, often the causative oncogenic mutation, correlate with Mef2 family members. The functional differences between the different Mef2 homologues in humans are not well understood and the specific role each may play in the Notch synergy remains to be elucidated.

Our analysis clearly indicates that, in *Drosophila*, the underlying molecular mechanism of the Notch/Mef2 synergy relies on the direct upregulation of expression of the prototypical TNF ligand *egr* through the binding of Mef2 and Su(H), the effector of Notch signals, to regulatory sequences on the *egr* promoter. In *Drosophila*, *egr* is the only JNK ligand while in humans, the superfamily is large and includes the cytokines TNF α (TNF), TRAIL and RANKL which have been associated with tumour progression in numerous human cancers including breast (Balkwill and Mantovani (2001)). RANKL plays a key role in bone metastasis of breast cancer, and is the target of a therapeutically effective monoclonal antibody (Gonzalez-Suarez, 2011). In breast cancer cells, TNF α , which can signal through several pathways, including JNK and NF- κ B, affects proliferation and promotes invasion and metastasis (Rubio *et al*, 2006).

In human breast cancer, clinical relapse after initial treatment is almost always accompanied by metastatic spread and it is almost invariably lethal. ER – tumours tend to respond well to first-line chemotherapy, but a significant subset of these tumours recur. Recurrent ER – tumours are typically resistant to chemotherapy and radiation, and are highly lethal. Our data suggest that ER – tumours that recur but not ER – tumours that do not recur show significant positive correlation between NOTCH1 and all four MEF2 paralogues. Further, our data show that even within the recurrent subset, NOTCH1 expression predicts poor survival but MEF2 expression does not. While these observations do not establish causality, they are consistent with the hypothesis that NOTCH1/MEF2 coexpression identifies a set of breast cancers that are more likely to relapse, and that MEF2 genes act as NOTCH cofactors rather than independently of NOTCH.

In conclusion, our study in *Drosophila* uncovers a new functional role for Mef2, which in synergy with Notch affects proliferation and metastasis. Mechanistically, this synergy relies on the direct upregulation of the JNK pathway ligand *egr*. The correlation analysis and tumour staining of human cancer samples suggests that our observations in *Drosophila* may well be valid in humans, defining Notch–Mef2 synergy as a critical oncogenic parameter, one that may be associated with metastatic behaviour, emphasizing the value of model systems in gaining insight into human pathobiology.

Materials and methods

Fly stocks

Fly culture and crosses were carried out at 25°C. *white*¹¹¹⁸ was used as wild type. UASN^{act}/TubPGAL80 Cyo; *E1Gal4* (*eyGal4*) virgins were crossed to males from the Exelixis collection (Thibault *et al*, 2004) for the modifier screen. Exelixis Mef2 lines were d06622 and

d03191. Other stocks used were UASN^{act}, UASMef2, UASGFP, UASbsk DN, UASeiger, UASp35, UASDiap1, *eiger*¹, and *puc*^{E69}. We also used *E1Gal4* (gift of G Rubin), *vgGal4*, *MS1096Gal4*, *hsp70-Flp*, *Actin5cGal4*>*y*+>*Gal4*, and *dppGal4*. Genetic clones were generated using using hsf1p; *Actin5C*>*y*+>*Gal4*;UASLacZ (10', 37°C heat-shock 48–72 h AEL) for overexpression of various genes.

Immunohistochemistry and microscopy

EdU (5-ethynyl-2'-deoxyuridine) assays were performed to directly measure active DNA synthesis in third-instar imaginal discs using the Click-iT EdU Alexa Fluor 555 kit (Invitrogen). Imaginal discs were dissected from third-instar larvae and incubated in 0.1 mg/ml EdU in Schneider's media for 20 min at room temperature. Immediately after EdU incorporation, discs were fixed in 4% formaldehyde in PBS for 20 min. EdU detection was performed according to the kit directions. Antibody staining was performed after EdU detection was completed.

For antibody stainings, third-instar larvae were dissected in PBS and fixed with 4% formaldehyde in PBS for 20 min, then permeabilized and stained in PBSTx (1 × PBS, 5 mg/ml BSA, 0.1% Triton X-100). The following primary antibodies were used: mouse anti-elav (9F8A9, 1:50; Developmental Studies Hybridoma Bank, DSHB), mouse anti-wg (4D4, 1:400; DSHB), mouse anti-MMP1 (3A6B4, 1:100; DSHB), mouse anti- β -integrin (CF.6G11, 1:100; DSHB), mouse anti-engrailed (4D9, 1:10; DSHB), rabbit anti- β -Gal (1:1,500; Cappel), Alexa Fluor 488[®] phalloidin (1:100; Molecular probes), rabbit anti-eiger (1:300 gift from M Miura), and rabbit anti-cleaved caspase-3 (1:300; Cell Signaling Technology).

Secondary antibodies conjugated to Alexa-488, Alexa-594, or Alexa-568 (Molecular Probes) were used at a dilution of 1:1000. Samples were analysed either by widefield fluorescence microscopy or by confocal microscopy (Nikon TE2000 w/C1 Point Scanning Confocal) and images were minimally processed using Adobe Photoshop.

Electromobility shift assays

We performed electrophoretic mobility shift assays (EMSAs) to determine whether or not Su(H) and Mef2 can bind to the *eiger* promoter. EMSAs were performed using the DIG Gel Shift Kit, 2nd generation (Roche applied Science). A 200-bp oligo containing the (–668 to –467) promoter region upstream from the *eiger* gene containing putative Notch and Mef2-binding sites was used for EMSA along with protein extracts from wild-type wing discs or wing discs coexpressing N^{act} and Mef2 (5 μ g each). Mutated versions of the putative Notch and Mef2-binding sites in this 200 base pair sequence were synthesized by Genescript and used as negative controls. The Notch and Mef2-binding sites in the 200-bp region were mutated from GTGAGAA to ACACAGG and CTAATAA to AGGGGGGGC, respectively. Supershift assays were performed using 5 μ g protein extracts made from either wild-type wing discs or wing discs coexpressing N^{act} and Mef2 and 30-mer oligos encompassing either the Su(H) binding site (GTTTAAAGTGAGAAAAGAACCG GTAAATC) or one of the Mef2-binding sites (CCATCGGGCAA ATTACTAAAATACATAAG). Supershifts were performed using antibodies against N^{act} (9C6, 1:20, DSHB) and Mef2 (1:20; gift from Nguyen).

Gene expression studies on human breast tumour samples

Notch signalling has been associated with breast cancer; however, the relationship between MEF2 genes and breast cancer has not been reported. In this study we used two publicly available gene expression data sets obtained from the GEO database accession # GSE2034 and GSE7390. The two data sets included a total of 484 breast cancer patients. In all, 343 patients were ER+ while 141 were ER–. In all, 158 patients relapsed whereas 326 patients did not relapse. The goal of this analysis was to explore the potential relationship between the four NOTCH genes and the four MEF2 genes. Gene expression data were generated using the Affymetrix platform using Human Gene Chip U133A. Details of sample collection and characteristics including preparation have been described in detail by the data originators (Wang *et al*, 2005; Desmedt *et al*, 2007).

For gene expression data analysis, we partitioned the data into two subsets, ER+ and ER–, treating them as separate disease entities. On each subset of data, we analysed the correlations between the NOTCH ligands and the MEF2 genes. Within each

disease entity, we further stratified the patients into breast cancer patients who did not relapse and those who relapsed and performed correlation analysis between NOTCH ligands and MEF2 genes on each subset of data separately. Estimate of correlations and Kaplan–Meier survival analysis were performed using SAS Statistical package version 9.1 (SAS Cary, NC, USA). We corrected for multiple hypothesis testing using the false discovery rate (FDR) (Benjamini and Hochberg, 1995) when comparing expression profiles of the genes between disease entities and between disease states (relapse versus no relapse) within disease entities. We also performed resampling using permutation tests to accurately estimate the *P*-values. However, because of the small number of genes involved, and because the goal was to assess the strength or similarity/dissimilarity in expression between genes, we did not control for multiple testing in the correlation analysis.

Supplementary data

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

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Conflict of interest

The authors declare that they have no conflict of interest.

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