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Short Article

Identification and Regulation of a Molecular Module for Bleb-Based Cell Motility

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SUMMARY

Single-cell migration is a key process in development, homeostasis, and disease. Nevertheless, the control over basic cellular mechanisms directing cells into motile behavior in vivo is largely unknown. Here, we report on the identification of a minimal set of parameters the regulation of which confers proper morphology and cell motility. Zebrafish primordial germ cells rendered immotile by knockdown of Dead end, a negative regulator of miRNA function, were used as a platform for identifying processes restoring motility. We have defined myosin contractility, cell adhesion, and cortex properties as factors whose proper regulation is sufficient for restoring cell migration of this cell type. Tight control over the level of these cellular features, achieved through a balance between miRNA-430 function and the action of the RNA-binding protein Dead end, effectively transforms immotile primordial germ cells into polarized cells that actively migrate relative to cells in their environment.

INTRODUCTION

Cell migration is a critical process that is involved in gastrulation, the generation of organs, and in the maintenance and function of the organs. This process is also key to normal and abnormal immune responses and constitutes the basis for a range of pathological conditions (Borregaard, 2010; Friedl and Gilmour, 2009; Richardson and Lehmann, 2010; Rørth, 2009; Roussos et al., 2011; Solnica-Krezel, 2005). A fundamental issue in cell migration concerns the actual acquisition of cell motility, the ability of cells to change their position relative to neighboring cells. Determining the mechanisms controlling cell motility would thus contribute to the understanding of a range of events in normal development, homeostasis, and disease.

A useful model for studying single-cell migration in vivo is that of primordial germ cells (PGCs) in early zebrafish embryos (Raz, 2004). The signal guiding zebrafish PGCs toward their target, the chemokine Cxcl12a, and the mechanisms controlling its distribution have been identified (Boldajipour et al., 2008; Doitsidou et al., 2002). Furthermore, the processes contributing to cell motility have been extensively studied. These studies showed that zebrafish PGCs generate protrusions in the form of actin-free cellular extensions powered by hydrostatic pressure (Blaser et al., 2006), while generation of traction that allows the cells to move with respect to neighboring cells requires the function of the cell-cell adhesion molecule E-cadherin (Kardash et al., 2010). Whereas those processes appear to be important for efficient PGC migration, it is not clear whether they are sufficient for promoting cell motility. Furthermore, the understanding of the molecular mechanisms regulating these cellular features is currently lacking.

A protein whose function is especially important in this context is the RNA-binding protein Dead end (Dnd) (Weidinger et al., 2003). This protein was found to function, at least in part, by counteracting the action of microRNAs (miRNAs), facilitating the stabilization and translation of specific mRNAs (Kedde et al., 2007). Interestingly, Dnd was shown to be essential for PGC motility, as cells knocked down for its activity display striking morphological defects, such that they fail to generate protrusions and to migrate (Weidinger et al., 2003). These immotile PGCs thus constitute an attractive platform for defining the fundamental processes controlling the acquisition of cell motility in vivo.

In this work, we show that the morphology and the motility of the germ cells rely on the proper regulation of contractility, as well as of cell adhesion and on the control over cortex-membrane interaction. Specifically, we demonstrate that cells lacking Dnd exhibit reduced contractile activity on one hand and elevated levels of Annexin A5b, a scaffold calcium- and phospholipid-binding protein that could control membrane-cortex interaction on the other. In addition, in the absence of Dnd, expression levels of the transcriptional repressor Zeb1 are reduced, resulting in elevated levels of the cell adhesion molecule E-cadherin. Consistent with the idea that contractility, adhesion level, and cortex-membrane interaction constitute key elements in the cellular motility program, manipulation of these determinants was sufficient to direct cells toward motile

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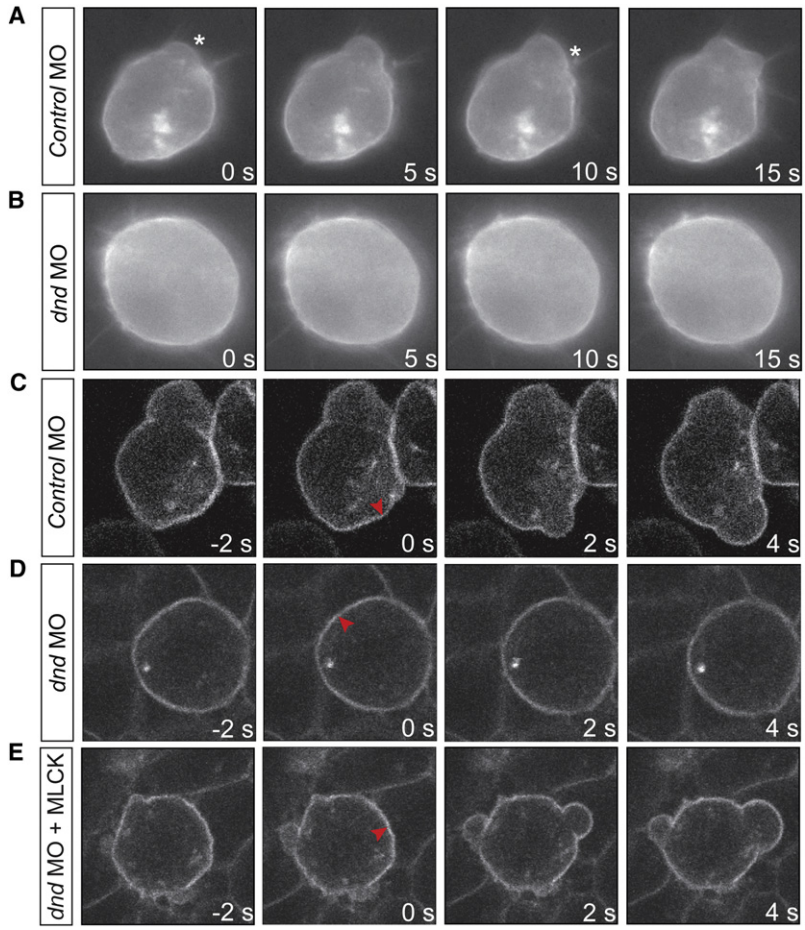

Figure 1. Lack of Bleb Formation in PGCs Knocked Down for Dnd Is Correlated with Reduced Contractility

(A) Control germ cells form blebs in the direction of migration. Asterisks mark bleb initiation.

(B) Lack of blebs in cells depleted for the Dnd protein.

(C) Cortex ablation induces blebs in control cells.

(D) Lack of bleb induction by a similar manipulation in cells knocked down for Dnd.

(E) MLCK restores bleb formation in response to cortex ablation.

Red arrowheads indicate the ablation point. See also Movies S1 and S2.

behavior. Specifically, enhancing the contractility in PGCs knocked down for Dnd, coupled to controlling the level of cell-cell adhesion and cortex properties, results in a striking recovery of protrusion generation and migration. Together, these findings define a set of cellular events that in concert potentiate single-cell motility in vivo and uncover the molecular mechanisms controlling them, namely, a balance between miRNA function and the action of a cell-specific RNA-binding protein.

RESULTS AND DISCUSSION

The Generation of Hydrostatic Pressure-Powered Protrusions in PGCs Depends on Dnd Function

During their migration, zebrafish PGCs dynamically generate cellular protrusions (Figure 1A; Movie S1, left section, available online) (Blaser et al., 2006). This cellular behavior depends on the function of the RNA-binding protein Dnd, as cells lacking it exhibit simple, round morphology and are immotile (Figure 1B and Movie S1, right section) (Weidinger et al., 2003).

Previous analysis of the protrusions generated by PGCs revealed that they are powered by hydrostatic pressure that pushes the membrane away from the actin cortex of the cell (Blaser et al., 2006). These protrusions, termed blebs, are produced by a range of other cell types (reviewed in Fackler and Grosse, 2008) and are nucleated at a point around the cell perimeter, where the interaction between the cell membrane and the cortex is reduced. At this position, local detachment of the membrane from the cell cortex occurs, followed by cytoplasmic flow-driven inflation, powered by intracellular pressure (Charras and Paluch, 2008). Indeed, similar to results obtained in vitro (Tinevez et al., 2009), using two-photon laser ablation to generate local damage to the cortex of PGCs in the context of the live embryo, we could effectively induce the formation of a bleb at the ablation point (Figure 1C and Movie S2, first section, 24 successful bleb inductions in 27 trials in 20 cells). Remarkably, a similar treatment of PGCs knocked down for Dnd function never resulted in bleb formation (Figure 1D and Movie S2, second section, no bleb induction in 48 attempts in 29 cells). Increasing the laser power resulted in a complete rupture of the cell (Movie S2, second section). These results are consistent with the idea that unlike wild-type cells, where intracellular pressure inflates the bleb, Dnd-depleted cells do not produce sufficient pressure for pushing the membrane away from the damaged cortex. Indeed, the simple round morphology of the Dnd knocked down PGCs is highly reminiscent of that observed in cells in which myosin contractility is inhibited (Blaser et al., 2006). To test this supposition, we introduced myosin light chain kinase (MLCK, referred to as MYLKa in zebrafish) into these cells, effectively restoring their ability to form blebs, in particular in response to laser-mediated damage to the cortex (Figure 1E and Movie S2, third section, induction of blebs in 24 out of 29 trials in 18 cells). Along these lines, overexpression of ROCK2a or RhoA, other activators of myosin contractility led to similar results (data not shown). Together, these experiments show that increasing myosin contractility, a feature connected to enhanced intracellular pressure (Charras et al., 2005; Sedzinski et al., 2011), is sufficient for restoring bleb formation in PGCs depleted of Dnd function. These observations are consistent with the notion that contractility-dependent hydrostatic pressure is at the basis of the characteristic morphology of wild-type motile PGCs. PGCs thus offer a useful model for exploring the genetic regulation over cellular hydrostatic pressure, a process of general importance in the control of cell shape and behavior (Charras et al., 2005; Stewart et al., 2011).

MicroRNAs Control MLCK Expression Levels

As the Dnd protein was shown to counteract miRNA function (Kedde et al., 2007), we set out to examine the possibility that the level of proteins controlling myosin contractility is regulated by miRNAs and Dnd.

We found that whereas *rhoA* lacks canonical seed sequences indicative of miRNA regulation, such elements are found in the 3′ untranslated regions (UTRs) of *rock2a* and *mlck.* To determine whether these mRNAs are subjected to miRNA regulation, we employed RNA reporters containing the green fluorescent protein (GFP) open reading frame (ORF) fused to the 3′ UTR of the *mlck* or *rock2a* RNA (or to that of the *nanos* RNA as an miRNA-responsive control) and injected it into 1-cell stage wild-type or MZ*dicer* mutant embryos that are devoid of mature miRNAs (Giraldez et al., 2005). Quantitative pixel-intensity analysis revealed dramatically elevated GFP level in somatic cells of 16 hr postfertilization (hpf) MZ*dicer* embryos as compared to wild-type control embryos in the case of *mlck*, but not for *rock2a* (Figures 2A, S1A, and S1B; data not shown). Thus, as previously shown for the *nanos* 3′ UTR (Mishima et al., 2006), the 3′ UTR of *mlck* could potentially confer negative regulation on the translation of linked ORFs through the action of miRNAs. Consistently, quantitative PCR (qPCR) performed on RNA extracted from MZ*dicer* and wild-type embryos revealed highly significant elevation in the endogenous levels of both *mlck* and *nanos* in embryos lacking mature miRNAs (Figure 2B). Interestingly, whereas *cis*-elements located within the *mlck* 3′ UTR lead to a reduction in *mlck* RNA levels and repressed GFP expression in somatic cells, the *mlck* 3′ UTR-containing RNA directed strong GFP expression to the PGCs (Figure S1C, inset).

Indeed, we found that *mlck* RNA possesses miRNA-430 binding sites in its 3′ UTR (Figure 2C), prompting us to assess the role of this miRNA in controlling MLCK level and in acquiring proper cell morphology and attaining motility. We analyzed the function of the three seed sequences for miR-430 (Figure 2C, blue boxes) by introducing point mutations within the seed sequences (Figure 2C, red boxes), or by application of "target protector" morpholinos (TP) (Figure 2C, *TP1*, *TP2*, *TP3*) (Choi et al., 2007). Both manipulations led to a strong increase in GFP expression, similar to that observed in MZ*dicer* embryos (Figures 2D, 2E, and S1C–1F). These results further substantiate the notion that *mlck* RNA is inhibited by miR-430 in the soma of zebrafish embryos.

Binding of Dnd to *mlck* mRNA Alleviates miRNA-Mediated Repression in Primordial Germ Cells

The results presented above indicate that MLCK expression and thus MLCK-dependent contractility is repressed in somatic cells by miRNAs. However, in the PGCs this repression was not effective (Figure S1C, inset), suggesting that miRNA activity on the 3′ UTR of *mlck* is alleviated in these cells. An attractive candidate to counteract the action of miRNAs in the PGCs is the Dnd protein that exerts such an effect on other RNAs like those encoding for Nanos and Tdrd7 (Kedde et al., 2007), proteins that are essential for germline development (Lehmann and Nüsslein-Volhard, 1991).

Indeed, Dnd function in the PGCs was essential for the expression of a fluorescent reporter whose ORF was fused to the wild-type 3′ UTR of the *mlck* RNA (Figures S1G and S1H). Importantly, the function of Dnd was dispensable when a miR-mutated *mlck* 3′ UTR was examined (Figures S1G and S1H). These findings suggest that Dnd counteracts the miR-430-mediated repression over *mlck* 3′ UTR in PGCs, explaining at least in part the enhanced contractility and dynamic cell-shape changes required for the motility of these cells. In agreement with this assertion, is the presence of Uridine-rich regions (URRs) within the 3′ UTR of *mlck,* sequences with which Dnd was shown to interact (Kedde et al., 2007) and which reside in close proximity to miRNA seed 1 (Figure 2F, violet boxes). As shown in Figures 2G and 2H, mutating these URRs resulted in a dramatic reduction in the level of the CFP reporter (CFP whose ORF was fused to this 3′ UTR). Consistent with such a direct role for Dnd in controlling the function of *mlck* RNA, we could demonstrate a specific interaction of Dnd with *mlck* RNA (Figures 2I and S1I). Together, these findings suggest that Dnd counteracts miR-430-mediated repression in PGCs by binding to URRs located within the *mlck* 3′ UTR, thereby controlling the cellular features relevant for bleb-associated motility. Increasing MLCK level in Dnd knocked-down cells restores bleb formation, but not motility. We therefore sought to identify additional molecules the function of which, in concert with that of MLCK would support active migration of the cells.

Regulation of Cell Adhesion by Dnd-Mediated Control of Zeb1 Expression

In addition to actomyosin contractility, the motility of zebrafish PGCs requires proper regulation of adhesion to surrounding cells through E-cadherin (Kardash et al., 2010). Interestingly, PGCs knocked down for Dnd, show elevated levels of E-cadherin, relative to those detected in migrating wild-type PGCs at the same developmental stage (Blaser et al., 2005). In addition, a slight reduction in E-cadherin level in wild-type PGCs precedes the acquisition of motility and is thought to allow the detachment of PGCs from neighboring cells, while maintaining sufficient level of the molecule compatible with the generation of traction (Blaser et al., 2005). In other cell types, E-cadherin was shown to be regulated at the transcriptional level as well as at the level of protein activity and localization (Ahn et al., 2011; Jeanes et al., 2008; Málaga-Trillo et al., 2009; Ulrich et al., 2005).

A plausible candidate for controlling E-cadherin expression in the PGCs is the transcriptional repressor Zeb1 (also named ZFHX1), which was shown to function in controlling epithelial-to-mesenchymal transition (Peinado et al., 2007). Relevant for this study, it was shown that Zeb1 expression is regulated by miRNAs in various cell types (Brabletz and Brabletz, 2010). We examined the miRNA regulation of Zeb1 expression in zebrafish by monitoring wild-type and MZ*dicer* embryos for 1. The fluorescent intensity of a GFP reporter construct containing *zeb1* 3′ UTR and 2. The endogenous *zeb1* mRNA level. Indeed, both parameters reveal that miRNAs interfere with Zeb1 expression, as a significant increase in GFP and *zeb1* mRNA level was observed in embryos lacking mature miRNAs (Figures 3A, 3B, and S2A). Consistent with these results, is the presence of a miR-430 binding site in the *zeb1* 3′ UTR (Figure 3C, blue box). To determine the relevance of this seed sequence for miRNA regulation, we examined the activity of reporter constructs in which the seed was mutated (Figure 3C, red box), or blocked by TP morpholinos (Figure 3C, *TP*). Both manipulations resulted

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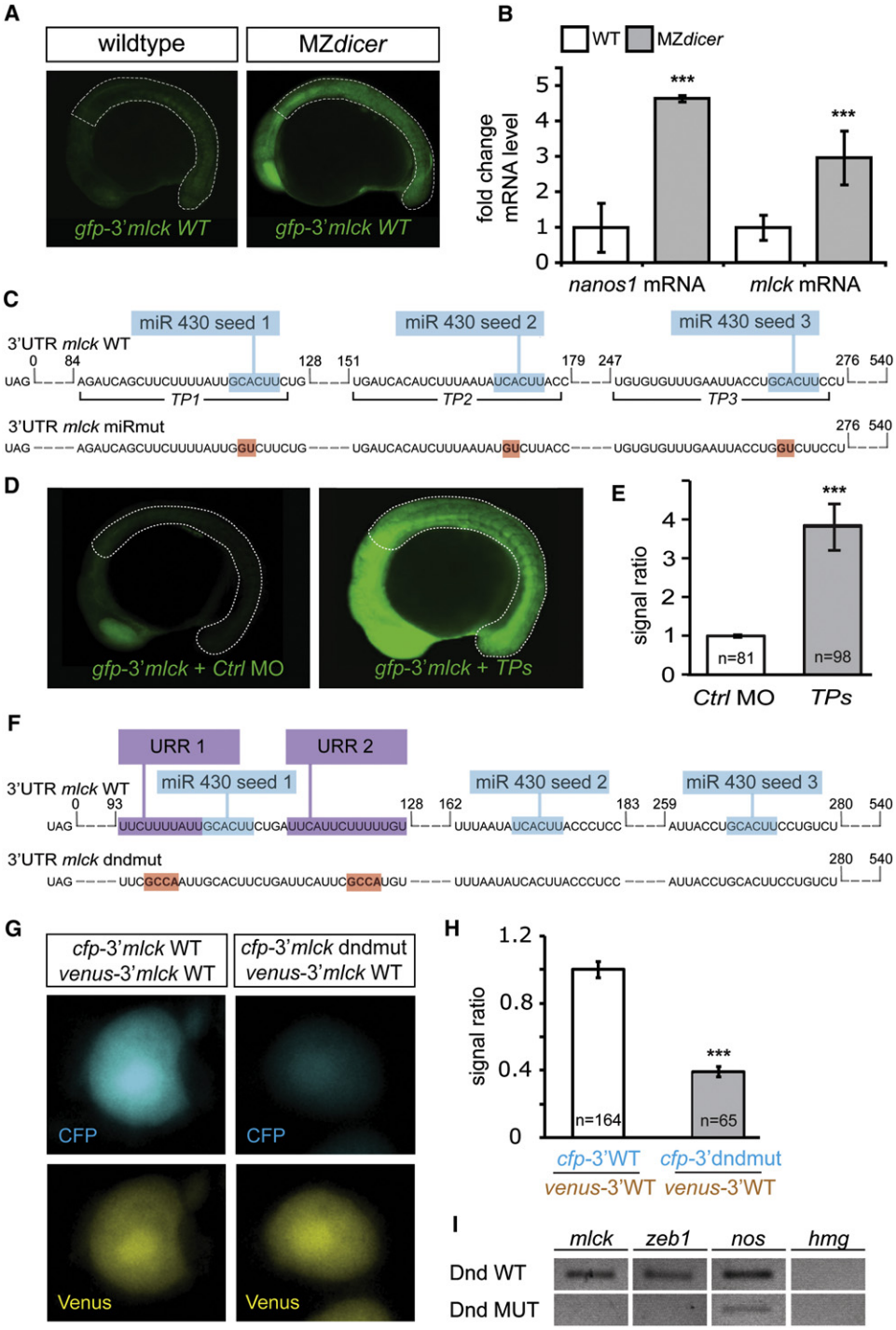

Figure 2. Control of MLCK Expression by microRNAs and Dnd Protein

(A) Lack of miRNAs in MZ*dicer* embryos results in higher GFP signal from a reporter containing the 3′ UTR of *mlck,* measured at 16 hpf within the dotted line marked areas (for quantitation, see Figure S1B).

(B) qPCR analysis comparing the endogenous levels of *mlck* and *nanos* mRNAs in wild-type and MZ*dicer* embryos.

(C) The 3′ UTR of *mlck* contains seed sequences for miR-430 (blue). Binding sites for target protector (TP) morpholinos that mask the miRNA seeds (*TP1-3*) are indicated and point mutations disrupting the seeds are labeled in red.

(D) Application of TPs increases GFP expression from the reporter RNA as compared to embryos treated with control morpholinos.

(E) Quantitative representation of the results of the experiment in (D), depicting the GFP signal level within the dotted line in (D) normalized to that of a coinjected mCherry control.

(F) The 3′ UTR of *mlck* contains two putative URR sites for Dnd binding (violet) adjacent to miR-430 seed sequences (blue). Mutations introduced are marked in red.

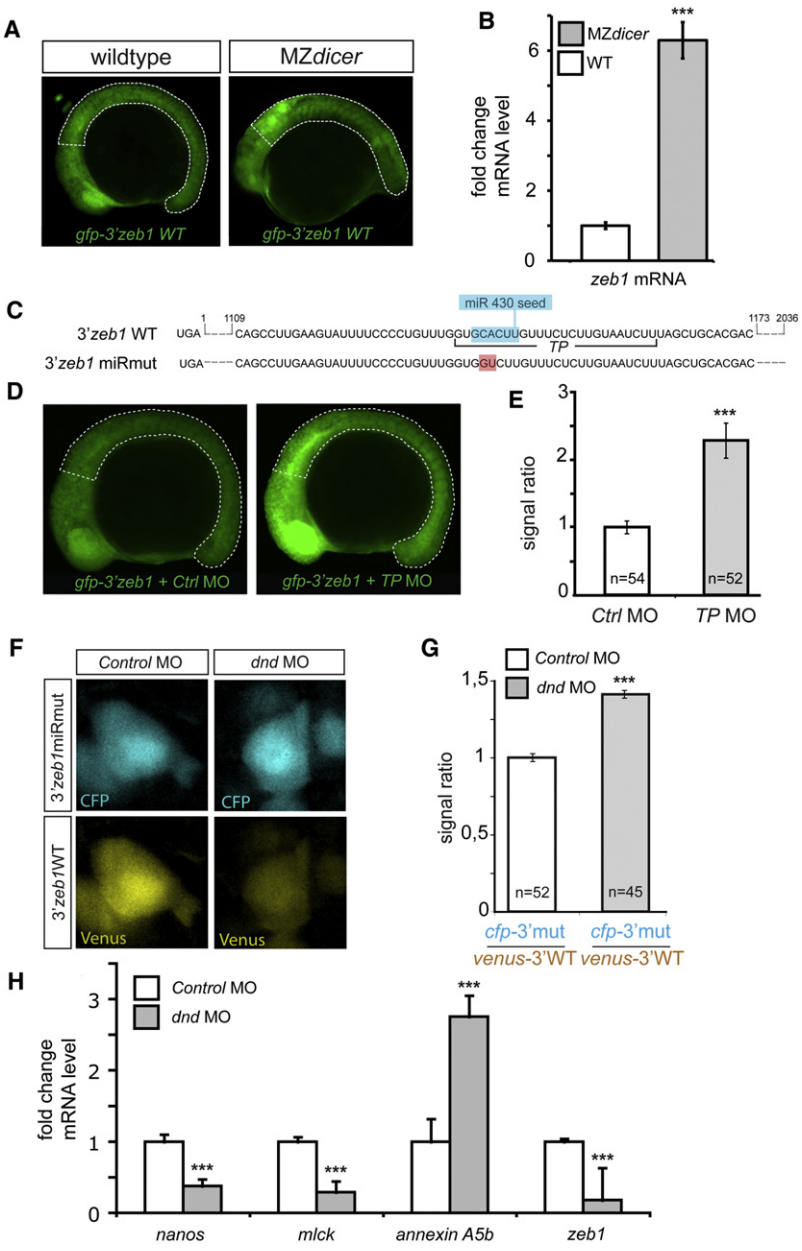

Figure 3. Control of ZEB1 and Annexin A5b Expression by miRNAs and Dnd

(A) Lack of miRNAs in MZ*dicer* embryos results in higher GFP signal from a reporter containing the 3′ UTR of *zeb1,* measured at 16 hpf from the line marked areas (Figure S2A).

(B) qPCR analysis comparing the endogenous levels of *zeb1* mRNA in MZ*dicer* and wild-type embryos.

(C) The 3′ UTR of *zeb1* contains a seed sequence of miR-430 (blue). Binding site for the target protector (TP) morpholino masking the miRNA seed (*TP*) is indicated and the point mutation disrupting the seed is labeled in red.

(D) Application of TPs increases GFP expression from the reporter RNA as compared to embryos treated with control morpholinos.

(E) Quantitative representation of the results of the experiment in (D) measured within the area marked by the dotted lines in (D), depicting the GFP signal level normalized to that of a coinjected mCherry control.

(F) Expression of the Venus protein from RNA containing the wild-type *zeb1* 3′ UTR is reduced in *dnd* MO treated PGCs as compared to that from an RNA reporter mutated for miRNA binding (*cfp-3′zeb1 miRmut*).

(G) Quantitative representation of the results in (F).

(H) qPCR analysis of endogenous *annexin* A5b, *nanos*, *mlck*, and *zeb1* mRNA in PGCs isolated from Dnd knocked-down embryos compared to control morpholino-injected animals.

n is the number of embryos analyzed in (E) or the number of PGCs examined in (G). Error bars depict the SEM and ***$p < 0.001$, calculated using two-tailed, unpaired t test. See also Figure S2.

in a strong increase in GFP level from the reporter construct (Figures 3D, 3E, and S2B), proving that miR-430 family members target *zeb1* 3′ UTR.

The miRNA-mediated regulation of Zeb1 in somatic cells could account for the higher level of E-cadherin detected in these cells, as compared to that observed in motile PGCs. We sought to determine whether in analogy to the control over contractility, Dnd protein is involved in controlling E-cadherin level in PGCs by regulating the expression levels of its repressor, Zeb1. In agreement with this notion, we found that the expression level of the reporter protein, whose ORF was fused to the 3′ UTR of *zeb1*, depended on Dnd expression in the PGCs (Venus in Figures 3F and 3G). Furthermore, the role of Dnd here is to counteract the action of miRNAs on the 3′ UTR of *zeb1*, since mutating the miR-430 seed rendered the reporter RNA insensitive to the lack of Dnd (CFP in Figures 3F and 3G). These results can be explained by the binding of Dnd protein to *zeb1* mRNA, as demonstrated in Figures 2I and S1I. Together, these findings suggest that Dnd functions in the PGCs to inhibit microRNA function on the mRNA encoding the transcriptional repressor Zeb1. This action of Dnd could in turn reduce the level

(G) Mutating the URRs in *mlck* 3′ UTR results in reduced CFP expression in the PGCs as compared to the control wild-type 3′ UTR. The coinjected RNA (*venus-3′mlck* WT) served as a control.

(H) Quantitative representation of the normalized signal intensity in the experiment presented in (G).

(I) Immunoprecipitation of Dnd protein followed by RT-PCR for bound RNAs showing binding of Dnd to *mlck* and *zeb1* compared to that of a Dnd version impaired for RNA binding (Dnd.MUT; Y104C, Slanchev et al., 2009). Amplification of *nanos* and *hmg* transcripts served as control.

n signifies the number of PGCs examined in (H) or the number of embryos analyzed in (E). Error bars depict the SEM and the ***$p < 0.001$, calculated using two-tailed, unpaired t test.

See also Figure S1.

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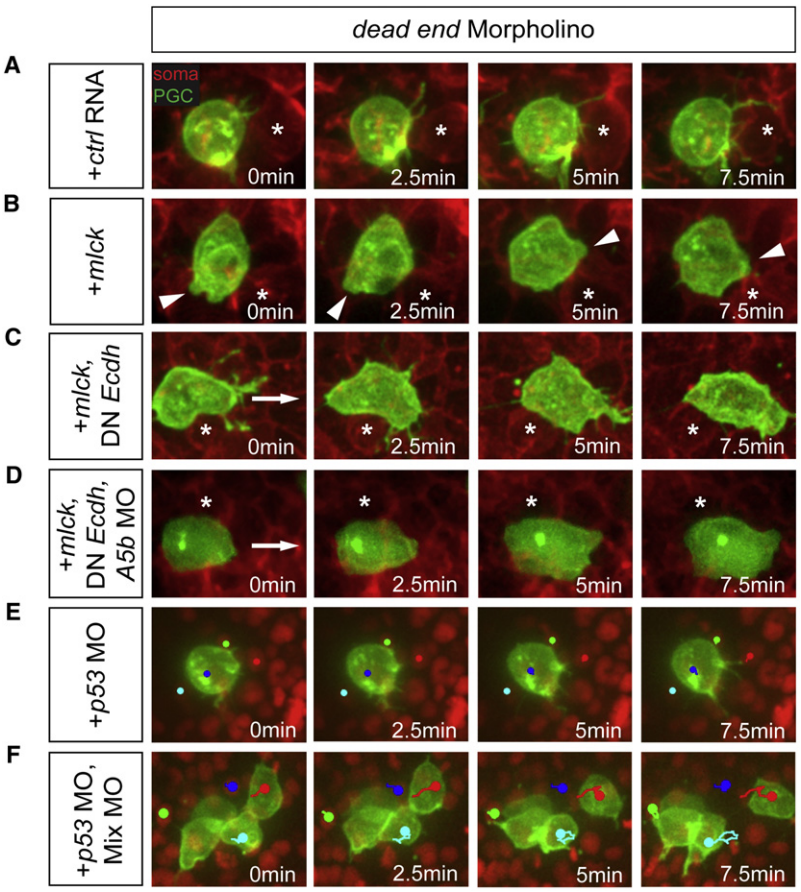

Figure 4. Motility Reprogramming of PGCs and Somatic Cells

(A) *dnd* MO treated PGCs (green) are immotile with respect to somatic cells (red).

(B) Bleb formation in immotile *dnd* MO treated PGCs overexpressing MLCK.

(C) Motility of *dnd* MO-treated PGCs expressing MLCK and a dominant-negative E-cadherin.

(D) Enhanced motility of *dnd* MO-treated PGCs knocked down for Annexin A5b in addition to the manipulation in (C).

(E and F) Dnd-depleted PGCs treated with p53 morpholino (E). Tracking a nucleus of such a round PGC (blue track) reveals no movement relative to somatic cells (light blue, green, and red). Such cells treated with a morpholino mixture (F), show active migration (red and light blue tracks for PGCs, green and blue for somatic cells) and cell shape changes.

Asterisks mark a stationary reference point in the soma. Arrowheads point at bleb formation positions. Arrows indicate direction of movement.

See also Figure S3 and Movies S4 and S5.

of E-cadherin in the PGCs, thereby promoting their motility. Accordingly, overexpression of Zeb1 resulted in overall lower E-cadherin protein levels in zebrafish embryos (Figure S2C). These results thus suggest that Dnd functions in regulating the adhesion level of the PGCs to cells in their environment, thereby promoting their motility by protecting *zeb1* mRNA from miR-430-mediated inhibition.

Downregulation of Annexin A5b Allows Bleb Formation in *dnd*-Morpholino-Treated PGCs

Annexins (ANXs) constitute a family of calcium- and phospholipid-binding proteins that have been implicated in a wide spectrum of cellular processes (Gerke and Moss, 2002; Rescher and Gerke, 2004). Included in these processes, Annexins are thought to control membrane to cortex attachment (Babiychuk et al., 1999; Bouter et al., 2011), a parameter considered to negatively regulate bleb formation (Diz-Muñoz et al., 2010; Lorentzen et al., 2011). Consistently, a dramatic increase in the mRNA encoding for Annexin A5b is observed in Dnd morphant PGCs, cells that do not bleb (Figure 3H). The increase in *annexin* A5b RNA levels in *dnd* morpholino-treated PGCs could be indirect and presumably miRNA independent. Irrespective of the mode by which Dnd controls Annexin A5b expression, we examined whether this scaffold protein could contribute to the lack of blebs in immotile Dnd knocked-down cells. Interestingly, downregulation of Annexin A5b in Dnd morphant embryos effectively restored bleb formation in PGCs (Figures S3A and S3B; Movie S3). Collectively, these results suggest that Annexin A5b-dependent cortex properties contribute to the Dnd knockdown phenotype and thus constitutes one of the components responsible for the rounded shape of these cells and their inability to move.

Reprogramming Bleb-Based Single-Cell Motility of PGCs

We have identified three key cellular properties that are differentially controlled in somatic and germ cells and showed that they are required for the acquisition of proper cellular morphology and motility. The importance of regulating these features in somatic cells is revealed by the fact that relieving *mlck* RNA from miRNA regulation (Figures S2D and S2E), overexpression of Zeb1 (Muraoka et al., 2000) and morpholino-mediated knockdown of Annexin A5b (Figure S2F) lead to defects in gastrulation. In the germ cells, manipulations of hydrostatic pressure, cell-cell adhesion, and cortex-membrane interaction affected the morphology of Dnd knocked-down cells, but individually were insufficient to restore motility. As cell movement involves the integration of several physical cellular properties, we next examined whether a combination of these could restore the motility of PGCs compromised for Dnd function.

In wild-type PGCs, Dnd enhances contractility by counteracting miR-430 action on *mlck* RNA. Indeed, increasing the contractility by reintroducing MLCK, effectively restored bleb formation in Dnd knocked-down cells that nevertheless, remained immotile (Figures 4A and 4B; Movie S4 first and second section). Further, by regulating Zeb1 level, Dnd also acts to reduce cell-cell adhesion to a level compatible with single-cell motility in the embryo. Last, Dnd function is important for controlling the cell cortex properties by maintaining low levels of Annexin A5b. In this case too, Dnd knocked-down PGCs in which the level of Annexin A5b was lowered exhibit dynamic alteration in cell morphology and formation of blebs, but remain immotile (Figure S3B; Movie S3).

These results thus correlate three cellular features, namely, myosin-mediated contractility, cell adhesion, and cell cortex-membrane interaction, with the ability to acquire proper cellular morphology and motility. We next investigated whether the combination of these components is sufficient for conferring single-cell motility in PGCs knocked down for Dnd function. As a first step, we examined the effect of reducing cell adhesion combined with increased contractility in PGCs knocked down for Dnd. To this end, we expressed moderate levels of MLCK (lower than those restoring bleb formation), as well as of a dominant-negative form of E-cadherin (EcdhΔEC1-2) in the PGCs, and observed a modest rescue of the Dnd phenotype; similar to the example shown in Figure 4C and Movie S4, third section, out of 60 cells examined in 30 embryos, over 80% of Dnd knocked-down cells that otherwise exhibited simple static morphology and were completely immotile showed polarization and limited movement relative to their neighbors. Moreover, providing such partially rescued PGCs with morpholino against *annexin* A5b, further enhanced their protrusive activity (by 2.6-fold, and by 1.7-fold when only blebs formed at the direction of cell movement were considered), as well as their motility relative to neighboring somatic cells (Figure 4D; Movie S4, fourth section).

The results presented above demonstrate that modulating three cellular parameters by a combination of RNAs and antisense oligonucleotides is sufficient for transforming immotile cells into cells that actively migrate. Subsequently, we sought to perform reprogramming of cellular motility by altering the activity of a small set of transcripts endogenously expressed in the PGCs. For this purpose, we used a mixture of target protectors that counteract the miR-430 function on *mlck* and *zeb1* RNAs (*mlck* TP1;2;3 and *zeb1* TP) and morpholinos inhibiting the translation of *annexin* A5b (*annexin* A5b MO). In addition, we included morpholinos inhibiting p53 translation in the mixture to inhibit cell death resulting from loss of RNAs such as *nanos* in PGCs lacking Dnd function (Robu et al., 2007), thus allowing us to focus on the role of Dnd in PGC motility (Figure 4E). Remarkably, out of 60 PGCs treated in this way in 25 different embryos, over 80% showed protrusive behavior and 40% actively migrated (Figures 4E and 4F; Movie S5, first and second section). Despite the dramatic reversion of the Dnd motility phenotype, the migration of the rescued PGCs that lack the expression of proteins responsible for PGC identity was slower than that wild-type cells exhibit, prohibiting them from effectively reaching their target. Germ cells differ from somatic cells with respect to the regulation over actin-based structures relevant for migration, in their ability to respond to specific guidance cues and the modes of cell adhesion that govern their motility. Interestingly, despite these differences, employing the "PGC-motility module" on somatic cells resulted in enhanced cell-shape changes and protrusive activity, while actual motility was not observed (Figures S3C and S3D; Movie S5, third and fourth sections). The lack of full migration of somatic cells provided with the PGC-motility module highlights the relevance of the initial state of the cell with respect to this property. This phenomenon is analogous to that observed in reprogramming cell fate, where the initial cellular state determines the response of cells to the relevant manipulations (Hanna et al., 2010).

In conclusion, we have demonstrated that a proper regulation of three biophysical parameters is sufficient to reverse the immotile state of PGCs lacking Dnd. Intriguingly, these parameters were sufficient to induce cell-shape changes and protrusive activity, characteristic of motile single cells, also in cells that normally do not show this behavior. The identification of this set of requirements for single-cell motility is relevant for processes in normal development when cells delaminate from their tissue of origin, as well as in disease conditions (Baum et al., 2008; Clay and Halloran, 2011). For example, gene expression profiles of cancer cells revealed upregulation of RNAs encoding for proteins controlling contractility, coinciding with downregulation of genes controlling cell adhesion and membrane to cortex attachment (e.g., microarray profile GDS2545 in Yu et al., 2004, GDS2618 in Kreike et al., 2006, and GDS1965 in Hoek et al., 2004). Studying the biophysical aspects governing PGC motility and the regulation of these cellular properties is thus likely to shed light on a range of processes where single-cell migration is involved.

EXPERIMENTAL PROCEDURES

Zebrafish Strains

Zebrafish (*Danio rerio*) of the AB background and transgenic fish carrying the *Tol-kop-egfp-f-nanos* -3′ UTR transgene (Blaser et al., 2005) were used as wild-type fish. MZ*dicer* mutant embryos (Giraldez et al., 2005) were used to examine the effect of lack of mature miRNAs. The zebrafish were handled according to the law of the state of North Rhine-Westphalia, supervised by the veterinarian office of the city Münster.

RNA Expression Constructs and Injections

Capped sense mRNA was synthesized using the mMessageMachine kit (Ambion). One and a half to 2 nanoliters were microinjected into the yolk of 1-cell stage embryos, unless stated otherwise.

The zebrafish ORF and 3′ UTR of *mlck* (NM_001105682) and *zeb1* (NM_131709) were amplified from zebrafish cDNA and cloned into expression vectors.

To direct protein expression to the germ cells, the ORFs were fused to *nanos* 3′ UTR, or to their own 3′ UTR to mimic the endogenous protein expression. A list of constructs is provided in the Supplemental Experimental Procedures.

Cortex Ablation Experiments

Ablation experiments were performed using a setup described before (Maghelli and Tolić-Nørrelykke, 2008) on PGCs of 7–8 hpf embryos. Defined circular region of interest centered immediately internal to the membrane was ablated.

Pixel Intensity Measurements

Ratio of mGFP/mCherry and Venus/SECFP were performed as previously described (Kedde et al., 2007).

Motility Induction

Embryos were injected with 600 pg of *control* or *dnd* MO together with 400 pg of *mlck.mlck3′*UTRmiRmut1;2;3 or PA-*gfp.globin3′* UTR as control to demonstrate bleb formation in *dnd*-morpholino-treated PGCs. For manipulation of MLCK and E-cadherin, 400 pg of DN *EcdhΔEC1-2.nanos* 3′ UTR and 400 pg *mlck.mlck3′*UTR miRmut1;2;3, or 800 pg of a control RNA (PA-*gfp*.globin3′UTR) were used. PGCs membranes were labeled with enhanced green fluorescent protein (EGFP) and all cells membranes were labeled with mCherry (Figures 4A–4D).

Manipulation of MLCK, E-cadherin, and Annexin A5b levels was performed by injecting 800 pg of *mlck* TP1, *zeb1* TP, 400 pg of *mlck* TP2 and TP3, 800 pg of *annexin* A5b MO1 or MO2, and 2 ng of *p53* MO into embryos knocked down for Dnd. Membrane of PGCs labeled with EGFP and all nuclei were labeled with H2B-mCherry (Figures 4E and 4F). The effect of this mixture

without p53 morpholino on somatic cell behavior (Figures S3C and S3D) was examined by injecting half of the above amounts into one of the four central cells of the 16-cell stage embryos whose cell membranes was labeled with EGFP.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, Supplemental Experimental Procedures, and five movies and can be found with this article online at doi:10.1016/j.devcel.2012.05.007.

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