Cell. Mol. Life Sci. 66 (2009) 145 – 155
1420-682X/09/010145-11
DOI 10.1007/s00018-008-8527-y

Cellular and Molecular Life Sciences

Research Article

Loss of FrmA leads to increased cell-cell adhesion and impaired multi-cellular development of *Dictyostelium* cells

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Received 28 August 2008; received after revision 10 October 2008; accepted 21 October 2008
Online First 11 November 2008

Abstract. Cell-cell adhesion is a critical property of all multi-cellular organisms and its correct regulation is critical during development, differentiation, tissue building and maintenance, and many immune responses. The multi-talin-like FERM domain containing protein, FrmA, is required during starvation-induced multi-cellular development of *Dictyostelium* cells. Loss of FrmA leads to increased cell-cell adhesion and results in impaired multi-cellular development, slug migration and fruiting bodies. Further, mixing experiments show that FrmA null cells are excluded from the apex of wild-type mounds, to which cells that normally form the organising centre known as the tip sort. These data suggest a critical role for FrmA in regulating cell-cell adhesion, multi-cellular development and, in particular, the formation of the organising centre known as the tip.

Keywords. Adhesion, development, FERM, migration, *Dictyostelium.*

Introduction

Cell-cell adhesion is a critical property of multi-cellular organisms. Failure to regulate cell-cell adhesion correctly leads to impaired development, tissue building and maintenance, cell-cell differentiation and many immune responses [1, 2]. Furthermore, cell-cell adhesion is critical for cancer development and progression [3]. Thus, understanding the basic mechanisms that underlie cell-cell adhesion is critical to understanding these physiological and pathological processes.

Studying cell-cell adhesion in higher eukaryotes is complicated by a number of factors including signalling complexity, redundancy and poor genetic malleability. *Dictyostelium discoideum* is an extensively studied model organism where cell-cell adhesion can be studied in a less complicated signalling and genetic environment. *Dictyostelium* cells normally live in the soil and exist as single cells that prey on bacteria. Upon depletion of their food source, *Dictyostelium* cells initiate a survival response which, over a period of ~24 h, sees cells undergo terminal differentiation and form fruiting bodies consisting of dead stalk cells supporting a mass of spores. A number of distinct steps occur on the way to forming the fruiting body, including the chemotatic coming together of ~100 000 cells, the formation of streams and mounds, cell-cell differentiation, cell sorting of differentiating cells within the mound, the formation of a photosensitive and migrating multi-cellular structure known as a slug and finally, culmination of the slug into a fruiting body.

Starvation initially up-regulates the expression of genes required for chemotactic confluence of cells. Once cells are in close proximity to each other and start to make contact and form a multi-cellular structure, genes that regulate cell-cell adhesion also

start being expressed. At least four different cell-cell adhesion systems are employed by *Dictyostelium* cells after the onset of starvation. Two of these systems appear shortly after and a few hours after the onset of starvation and are defined by their requirement for Ca^{2+} or Mg^{2+}, respectively [4, 5]. While CadA is the first to appear and is responsible for the Ca^{2+}-mediated cell-cell adhesion system, the adhesion molecule responsible for the slightly later appearing Mg^{2+}-mediated cell-cell adhesion system, remains unknown. Once cells have started to form multi-cellular structures, *i.e.* streams, mounds slugs and fruiting bodies, the remaining cell-cell adhesion systems appear [6–9]. These adhesion systems are mediated by CsaA and LagC and are independent of Ca^{2+} and Mg^{2+}. The signalling events that regulate these cell-cell adhesion systems remain largely unknown.

The aim of this study was to determine the role of FrmA during starvation-induced multi-cellular development of *Dictyostelium* cells and, in particular, its role in cell-cell adhesion and development. FrmA is a multi-talin-like FERM domain containing protein that has previously been shown to negatively regulate cell-substrate adhesion of both non-starved and starved *Dictyostelium* cells [10]. Here we present data showing that loss of FrmA also leads to increased cell-cell adhesion and impaired starvation-induced multi-cellular development. Further, FrmA null cells are unable to efficiently sort to the apex of mounds and form the organising centre known as the tip.

Materials and methods

Strains used and culturing conditions. All strains used in this study are derived from Ax2 wild-type (wt) cells and maintained in HL-5 medium at pH 6.8 (ForMedium Ltd., HLG0102). GFP was expressed in wt and FrmA null (null) cells and FrmA, tagged with the HA epitope, was expressed in null cells. The null cells were maintained in 10 μg/ml Blasticidin, while cells expressing GFP or FrmA(HA) were maintained in 15 μg/ml of G418.

Development, slug migration and Neutral Red staining. Six-hour-starved cells were prepared by washing non-starved cells twice in phosphate buffer (10 mM KH_2PO_4, 10 mM K_2HPO_4, pH 6.8), re-suspending them in phosphate buffer at 1×10^7–2×10^7 cells/ml and shaken at 120 rpm for 6 h.

To record the multi-cellular development of cells, non-starved cells were washed twice in phosphate buffer and plated onto either phosphate-buffered or H_2O 1 % non-nutrient agar at 5×10^5 cells/cm^2. Individual images were captured using a standard Olympus IX81 inverted microscope with a 5× objective. For time-lapse video microscopy, phase and fluorescent images were captured every 15 min with a 5× objective using a standard inverted Olympus IX81 microscope run using ScanR software.

For slug migration, non-starved cells were washed twice in H_2O and spotted onto H_2O 1 % non-nutrient agar at 1×10^7 cells/ml. Cells were left to develop for up to 25–30 h in the presence of a unidirectional light source.

For Neutral Red staining, 1×10^7 cells in 1 ml H_2O were incubated for 45 s with 0.001 % Neutral Red (w/v in H_2O), washed twice in H_2O and spotted onto H_2O 1 % non-nutrient agar.

Measurement of cell-cell adhesion. Non-starved and 6-h-starved cells were washed once in phosphate buffer, thoroughly pipetted and re-suspended in phosphate buffer at a cell density of 1×10^7 cells/ml. The number of single cells was determined immediately or after rotating at 120 rpm for 30 or 60 min with or without 10 mM EDTA.

For non-starved cells, to take into account any cell division that may take place during 60 min of rotation, the number of single cells after treatment with 10 mM EDTA was used to normalise the value of single cells present in non-treated cells as no cell clumps were present. For starved cells, where there is no more cell division, the initial input of cells was used to normalise the values for single cells in non-treated cells after 30 and 60 min and in 10 mM EDTA treated cells after 60 min.

The aggregates formed by non-starved and 6-h-starved cells were rotated constantly for 60 min at 120 rpm before images were captured to record the size of aggregates formed. This was done in the absence of EDTA and the average size of the aggregates was determined using ImageJ analysis software.

Determination of gene expression levels. Total RNA was isolated (Qiagen, RNeasy Mini kit) from non-starved and cells starved for 8, 16 and 24 h. Real-time RT-PCR (Quantace, SensiMix One-Step Kit) was used to determine the expression levels of *Dd*FrmA, GFP, CadA CsaA, LagC, EcmA and CotA. The primers used for real-time RT-PCR: FrmA, 5′-ATTGGTGCAGCTGATGATTG-3′ and 5′-GACCCCACAATTCCAAACTG-3′; GFP-3′, 5′-AAGTCGTGCTGCTTCATGTG-3′ and 5′-ACGTAAACGGCCACAAGTTC-3′; CadA, 5′-TTCCAAGAATTGGCTCAAGG-3′ and 5′-TCATCACCATCTTTGCAAGC-3′; CsaA, 5′-TAACCATGGGAACCTCAAGC-3′ and

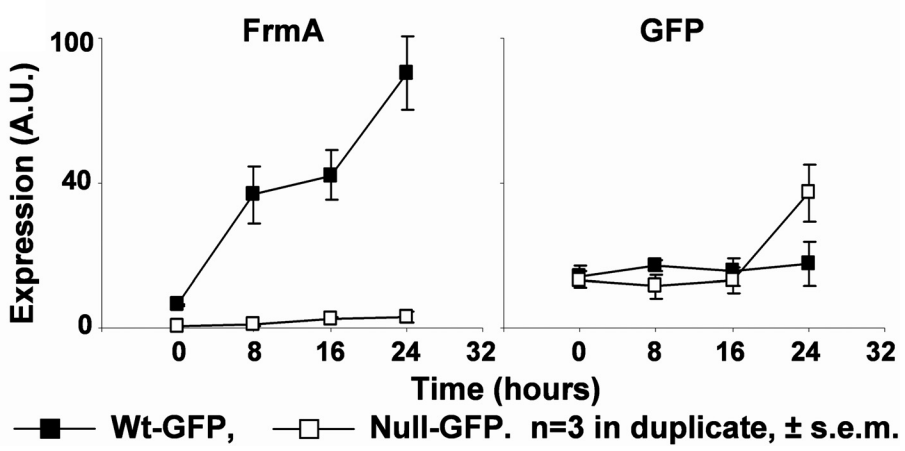

Figure 1. Expression of FrmA is present throughout starvation induced multi-cellular development. Non-starved wt-GFP and FrmA null-GFP (null-GFP) cells were starved on phosphate-buffered, non-nutrient agar for the indicated times and total RNA collected for real-time RT-PCR analysis. In wt-GFP cells (filled squares) expression of FrmA increases upon starvation and continues to do so throughout multicellular development (left). Expression of FrmA is lost in null-GFP cells (open squares). Expression of GFP was used as RNA loading control (right).

5′-ACAATCGAAACATGGGCATT-3′; LagC, 5′-CAGAACTGAAATGGCACCAA-3′ and 5′-AAACCCGAAGCATCAAATGT-3′; EcmA, 5′-TCGAACCAATCAAACTTCCA-3′ and 5′-ACCAGTCTTGGAATCGCAAC-3′; CotA, 5′-TGGTGGTGCATTAGCACAAT-3′ and 5′-CCATGTTTGGACTGGGAGAT-3′.

Results

FrmA expression increases upon starvation-induced multi-cellular development. We previously reported that the expression of FrmA transcript was up-regulated in wt Ax2 cells after 6-h starvation in phosphate buffer [10]. To establish whether FrmA transcript was expressed during later stages of starvation-induced multi-cellular development, log phase growing wt cells expressing GFP (wt-GFP) were washed once in phosphate buffer and starved on non-nutrient phosphate-buffered agar. At 0, 8, 16 and 24 h of starvation, total RNA was isolated and the level of FrmA transcript determined by real-time RT-PCR (Fig. 1A). Expression of FrmA transcript was increased upon starvation of wt-GFP cells and continued throughout development. Expression of FrmA is 5.7-, 6.5- and 10.9-fold higher at 8, 16 and 24 h, respectively, compared to expression at 0 h. The profile of FrmA expression was identical in wt and wt-GFP cells (data not shown) and for the remainder of this report wt-GFP and null-GFP cells were used for expression studies. Expression of GFP, which is expressed under the control of the Actin-15 promoter within an extrachomosomal plasmid, was used as an RNA loading control. Thus, FrmA transcript is present during all stages of starvation-induced multi-cellular development. FrmA protein levels could not be determined due to the unavailability of anti-FrmA specific antibody.

FrmA null (null) cells were previously used to characterise the effects of the loss of FrmA on cell-substrate adhesion and cell migration [10]. Like null cells, null cells expressing GFP (null-GFP) did not express FrmA as determined by real time RT-PCR (Fig. 1A) and these cells were used to describe the role of FrmA in starvation induced multi-cellular development.

Loss of FrmA impedes all stages of multi-cellular development. wt-GFP and null-GFP cells were starved on non-nutrient, phosphate-buffered agar and imaged at 10, 20 and 30 h (Fig. 2A). wt-GFP cells formed streams by 10 h of starvation, while null-GFP cells failed to do so (Fig. 2A). At 20 h, whereas wt-GFP cells had formed slugs and fruiting bodies, null-GFP cells had only reached the tight mound stage. At 30 h of development, all wt-GFP cells had formed fruiting bodies, while null-GFP cells had formed small slugs and stunted and/or malformed fruiting bodies (Fig. 2A, right panel of images). Expression of FrmA tagged with HA in null cells (null-FrmA^{HA}), rescued the delayed development of null cells as well as the wt morphology of the multi-cellular structures (Fig. 2A). The development of wt or null cells that express GFP was identical to the GFP non-expressing cells (data not shown). Time-lapse video microscopy was used to record the development of wt-GFP and null-GFP cells and showed that, typically, wt-GFP cells formed tight mounds by 12.5 h, tipped mounds by 15 h, slugs by 17.5 h and fruiting bodies by 20 h (Supplemental Movie 1, Supplemental Fig. 1). However, null-GFP cells only formed tight mounds after 17.5 h of starvation, slugs after 22.5 h and finally stunted and/or malformed fruiting bodies after 27.5 h (Supplemental Movie 2, Supplemental Fig. 1). The time taken between tight mound formation to slug formation, and from slug formation to fruiting body formation, was approxi-

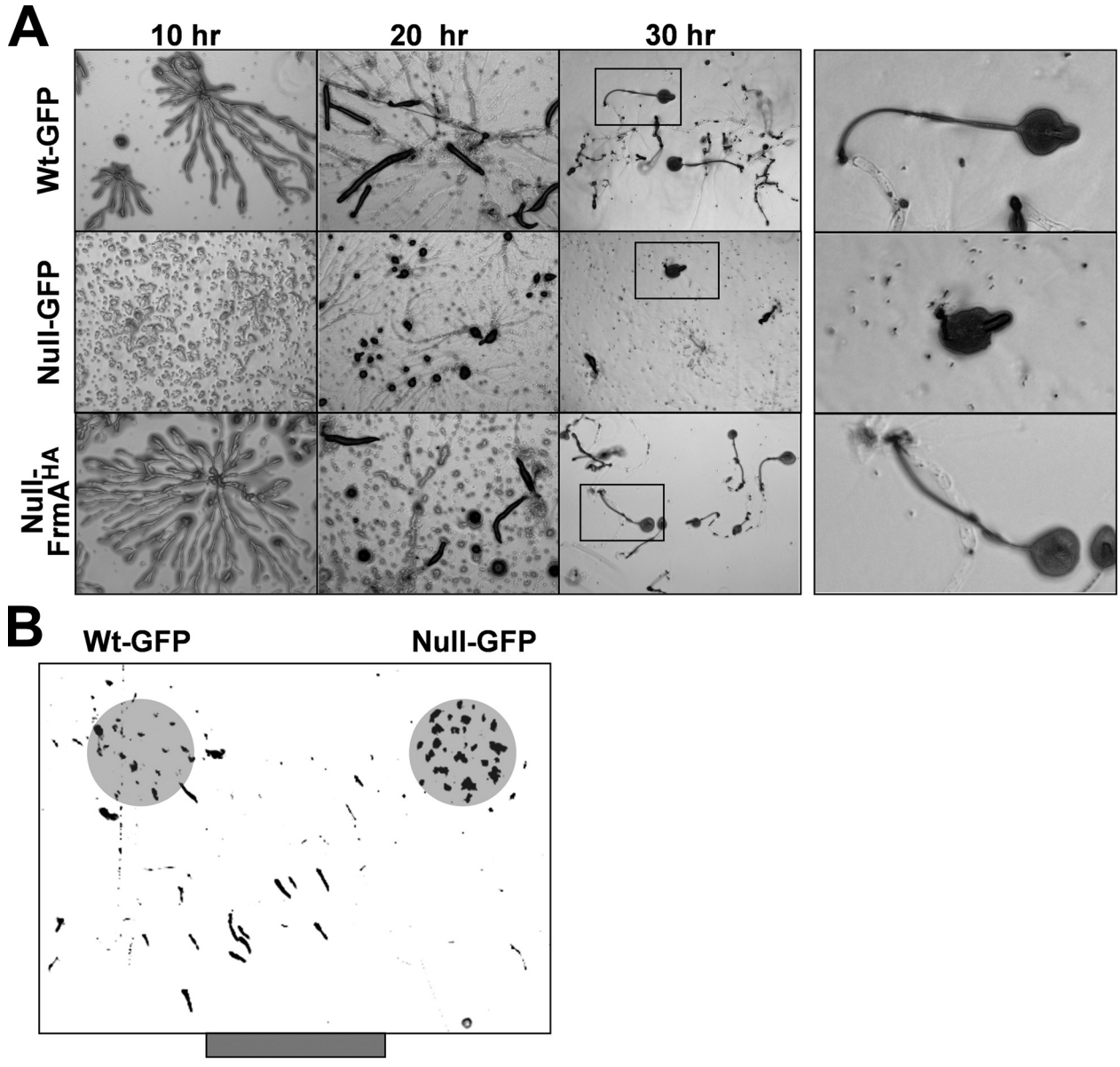

Figure 2. Multi-cellular development and slug migration are impaired in FrmA null-GFP (null-GFP) cells. Non-starved wt-GFP, null-GFP and null cells expressing full-length FrmA tagged with HA (null-FrmA^{HA}) were starved on phosphate-buffered, non-nutrient agar and images captured at the times indicated (*A*). Null-GFP cells were delayed in multi-cellular development and the fruiting bodies they produced were short and malformed (*A* and extreme right panel). Multi-cellular development of null-FrmA^{HA} cells was identical to wt-GFP cells. Wt-GFP and null-GFP cells were developed on un-buffered, non-nutrient agar to facilitate slug migration (*B*). Slugs formed by null-GFP cells did not migrate towards the light, whereas wt-GFP slugs did. Cells were spotted (grey circles), allowed to develop for 25 h in the presence of a unidirectional light source (grey rectangle) and imaged.

mately 2.5 h in wt cells. These periods were approximately doubled, to 5 h, in the case of null cells. Thus, loss of FrmA leads to a delay at all stages of multicellular development.

An important part of the starvation-induced survival response is to deliver potential spores to an environment that will maximise their chance of survival upon hatching. *Dictyostelium* cells achieve this at the slug stage where slugs are able to migrate in both a thermo-, photo- and chemo-sensitive manner [11, 12]. Slug migration involves the co-ordinated movement of a number of different cell types that have to both move as a whole and remain confined to specific areas within the slug as it migrates. Processes that are critical for achieving this include directed cell migration and cell-cell adhesion. To test the ability of wt and null slugs to migrate, non-starved wt-GFP and null-GFP cells were washed and spotted onto non-nutrient water agar plates and allowed to develop in the presence of a unidirectional light source (Fig. 2B). Whereas wt-

GFP cells formed slugs that migrated towards the light source after 25 h, none of the slugs formed by the null cells migrated towards the light. Even after 35 h, no null-GFP slugs had started to migrate in the direction of the light source (data not shown). In most cases, null-GFP slugs remained where they formed and culminated into malformed fruiting bodies.

Cell-cell adhesion and aggregate size are both increased in FrmA null cells. One possible explanation for the delays seen at the different stages of multicellular development of null cells and their inability to form migrating slugs could be due to deregulated cell-cell adhesion. To test this possibility, cell-cell adhesion assays were carried out with wt-GFP and null-GFP cells. Non-starved wt-GFP and null-GFP cells were washed once in phosphate buffer, disaggregated by thorough pipetting and constantly rotated at 120 rpm for 60 min with or without 10 mM EDTA (Fig. 3A and B, top graph). Treatment of non-starved cells with 10 mM EDTA prevented aggregate formation of both wt-GFP and null-GFP cells, as cells are thought to only acquire EDTA-resistant cell-cell adhesion after the onset of starvation. The number of single cells after 1 h of treatment with 10 mM EDTA was used to normalise the value for the number of single cells not treated with EDTA. Non-starved null-GFP cells consistently showed a greater ability to form aggregates than wt-GFP cells. After 60 min, 22.2 % of null-GFP cells remained as single cells, while 45.2 % of wt-GFP cells still remained as single cells.

Following starvation for 6 h, null-GFP cells also showed a greater ability to form aggregates than wt-GFP cells. At 30 min after initial disaggregation, only 26.0 % of null-GFP cells remained as single cells, while 55.5 % of wt-GFP cells remained as single cells. After 60 min, 27.2 % of null-GFP cells remained as single cells, while 39.9 % of wt-GFP cells remained as single cells. In contrast to non-starved cells, treatment with 10 mM EDTA did not completely prevent aggregate formation with null-GFP cells in particular showing an increased resistance to EDTA compared to wt-GFP cells. In the presence of EDTA, 41.7 % of null-GFP cells remained in aggregates compared with only 19.1 % of wt-GFP cells remaining in aggregates.

Another measure of cell-cell adhesion in *Dictyostelium* cells is the size of aggregates they form (Fig. 3C). Aggregates formed by null-GFP cells, at both the non-starved and 6-h-starved stages, were greater in size than the aggregates formed by wt-GFP cells. On average, the size of aggregates formed by non-starved and 6-h-starved null-GFP cells, was approximately 2.5- and 3-fold that of aggregates formed by wt cells, respectively. For wt-GFP cells, the average area of aggregates formed by non-starved cells was 1.3 mm^2, while for 6-h-starved cells, it was 0.9 mm^2. Thus, null-GFP cells showed greater cell-cell adhesion than wt-GFP cells, suggesting that their impairment in multicellular development may be due to increased cell-cell adhesion.

Expression of LagC is delayed in FrmA null cells, whereas differentiation is unaffected. The expression of a number of cell-cell adhesion molecules, such as CadA, CsaA and LagC, are all known to be regulated during starvation-induced multi-cellular development. To determine whether expression of these molecules was affected by the loss of FrmA, real-time RT-PCR was carried out. Total RNA was collected form non-starved cells and cells starved for 8, 16 and 24 h on phosphate-buffered agar for analysis (Fig. 4A). The largest difference in expression was seen for LagC at 8 h of development, with null-GFP cells (8.23 ± 4.76 A.U.) showing a significantly lower level of LagC expression compared to wt-GFP cells (99.08 ± 22.51 A.U.). Expression of CadA and CsaA at 8 h of development was also lower in null-GFP cells compared to wt-GFP cells but the magnitude of the difference and significance for both were much lower than for LagC.

We also looked at starvation induced differentiation markers. Once starving *Dictyostelium* cells start to form multi-cellular structures such as mounds, cells start to differentiate into two basic cell types that go on to form either spores or stalk cells. The two basic cell types are known as pre-spore and pre-stalk cells and make up 80 % and 20 % of the mound, respectively. Real-time RT-PCR was used to detect the expression levels of pre-stalk and pre-spore markers (EcmA and CotA, respectively) (Fig. 4B) at 0, 8, 16 and 24 h of multi-cellular development. Levels of EcmA and CotA were similar in wt-GFP and null-GFP cells (Fig. 4B).

FrmA null cells are excluded from sorting to the organising centre known as the tip. The largest difference in the expression of cell-cell adhesion molecules between wt-GFP and null-GFP cells was the reduced expression of LagC in null-GFP cells after 8 h of starvation. Cells that are null for LagC, arrest at the mound stage of multi-cellular development, fail to form tips and, when mixed with wt cells, accumulate within the rear portion of the pre-spore region [7, 13, 14]. After mound formation, cells that form the organising centre known as the tip, and are known as pre-stalk cells, form randomly within mounds and eventually sort to the apex of the mounds. FrmA null cells show a delay in transition from the mound stage to the slug stage and to determine whether this due to an inability to form a tip efficiently, mixing experi-

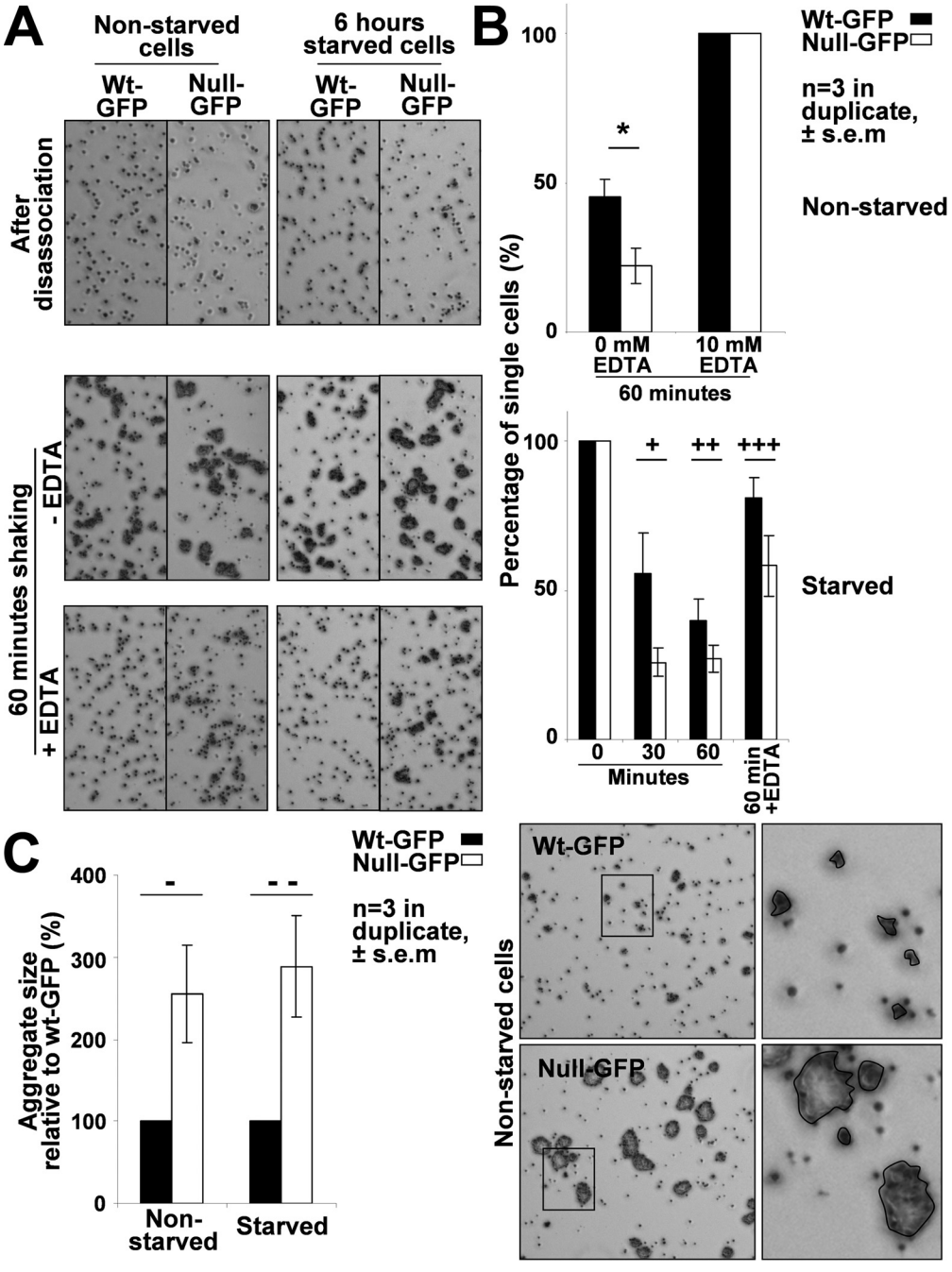

Figure 3. FrmA null-GFP (null-GFP) cells have increased cell-cell adhesion. Non-starved and 6-h-starved wt-GFP and null-GFP cells were washed, thoroughly dissociated and subjected to constant rotation at 120 rpm with or without 10 mM EDTA. The number of single wt-GFP (filled bars) and null-GFP (open bars) cells were determined at the times indicated. Representative images are shown (*A*) and the normalised averages of three experiments ± SEM. are shown (*B*, * $p<0.005$, $^{+}$ $p=0.046$, $^{++}$ $p=0.029$ and $^{+++}$ $p=0.016$). The average size of the aggregates formed by the wt-GFP (filled bars) and null-GFP (open bars) cells in the absence of EDTA were determined after 60 min of constant rotation at 120 rpm using ImageJ analysis software (*C*, $^{-}p < 0.001$ and $^{--}p<0.001$). Images of a typical field of wt-GFP and null-GFP cells after 60 min are shown (*C*, right) and magnified insets (boxes) are also shown (*C*, extreme right). Outlined within the magnified images are typical aggregates formed by the cells that were measured. Single cells were excluded from the measurements and groups of aggregates were differentiated where possible.

ments with wt cells were carried out. Briefly, 95 % wt or null cells were mixed with 5 % wt-GFP of null-GFP cells [Fig. 5, Supplemental Movie 2 ($wt^{95\%}$/wt-$GFP^{5\%}$), Supplemental Movie 3 ($wt^{95\%}$/null-$GFP^{5\%}$), Supplemental Movie 4 ($null^{95\%}$/wt-$GFP^{5\%}$) and Supplemental Movie 5 ($null^{95\%}$/null-$GFP^{5\%}$)]. The sorting of the GFP-expressing minority within the non-GFP expressing majority, is an indicator of how well the

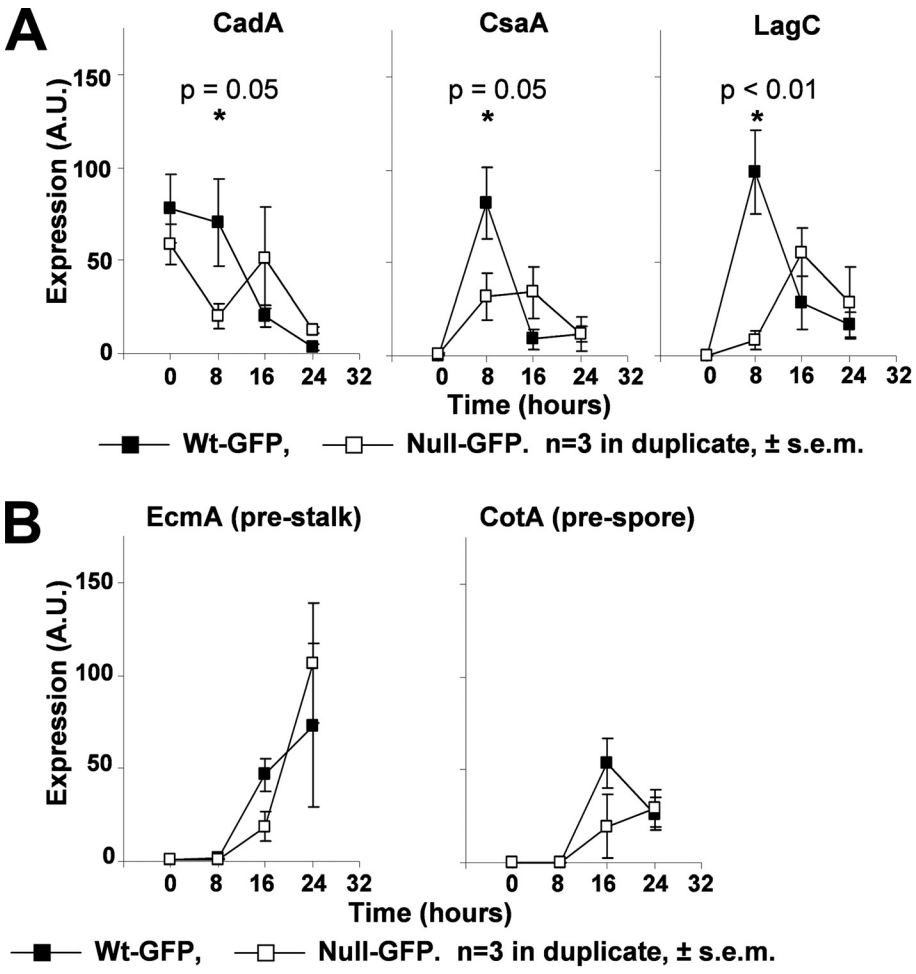

Figure 4. Expression of LagC is decreased at 8 hours of starvation in FrmA null-GFP cells (null-GFP), while differentiation is unaffected. Non-starved wt-GFP and null-GFP cells were starved on non-nutrient agar for the indicated times and total RNA collected for real-time RT-PCR analysis. Wt-GFP (solid squares) and null-GFP (open squares) cells showed small differences in the expression for CadA or CsaA (*A*). However, LagC expression was greatly reduced at 8 h of development. Differentiation markers EcmA and CotA are similar in wt-GFP and null-GFP cells throughout development (*B*).

minority respond to the global cues within the majority structures. If the GFP-expressing cells are equivalent to the non-GFP-expressing cells then as mounds and slugs start to form, the distribution of the GFP-expressing cells should be random. However, if there is a difference between the GFP- and non-GFP-expressing cells, then the GFP-expressing cells would preferentially sort to a specific area within mounds and slugs. This differential sorting could be the result of either a loss of function or a gain of function or even of having the ability to fulfil a function that is largely impaired. As expected, mounds made up of $wt^{95\%}$/wt-$GFP^{5\%}$ cells had a random distribution of wt-GFP cells within mounds (Fig. 5A). This shows that the expression of GFP had no effect on the ability of wt-GFP cells to respond to global cues within the majority wt tight mound. Similarly, even though the formation of mounds was delayed in $null^{95\%}$/null-$GFP^{5\%}$ cells, the distribution of null-GFP cells was random. However, the mixing of $wt^{95\%}$/null-$GFP^{5\%}$ led to null-GFP cells being excluded from the apex of mounds, *i.e.* the organising centre known as the tip. The reverse experiment sees the wt-GFP cells occupying the apex of predominantly null mounds. These data suggest that the null cells only reluctantly form tip cells compared to wt cells. Further, null cells are able to produce the global cues that facilitate sorting, as wt-GFP cells were able to sort to the apex of predominantly null mounds and form the tip (Fig. 5A). However, these tips rarely progressed on to form a migrating slug.

One possible reason for null cells being reluctant in sorting to tips within predominantly wt mounds could be due to their inability to migrate within mounds as wt cells. This may be similar to that seen to occur in 6-h-starved null cells, where migration of single null cells towards cAMP was slower than that of wt cells [10]. This was due to the increased cell-substrate adhesion of the null cells. Within multi-cellular structures, such as mounds, the movement of the null cells could also be similarly impaired but this time due to a defect in cell-cell adhesion. To determine whether this was the case, wt-GFP and null-GFP cells within predominantly wt mounds, were tracked and their paths determined (Fig. 5B). Whereas wt-GFP cells moved in a circular manner in one direction around the mound,

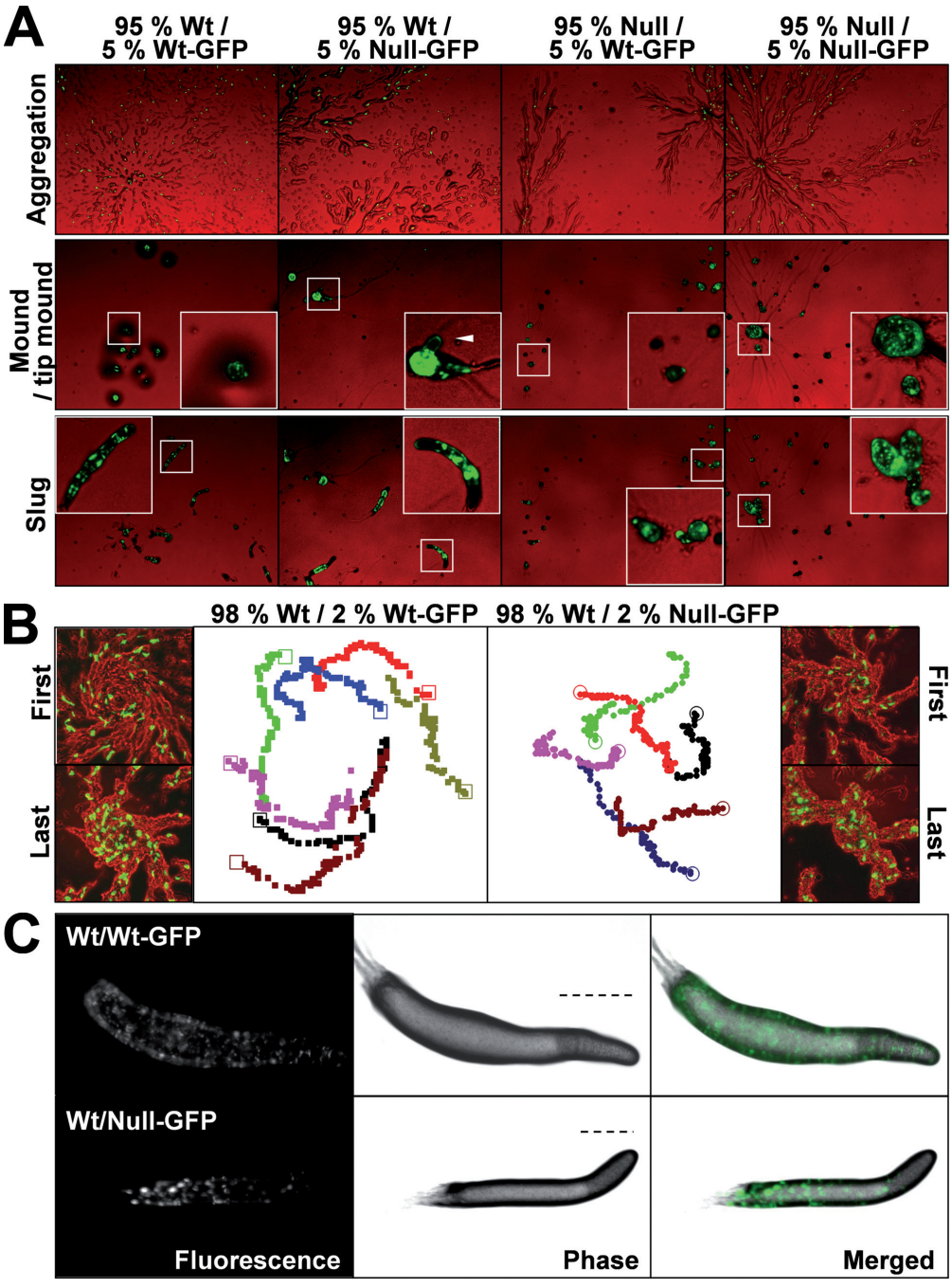

Figure 5. FrmA null cells are excluded from sorting to the organising centre known as the tip due to their impaired movement within mounds. Mixtures of cells consisting predominantly of GFP-non-expressing (95 % wt and null) and GFP-expressing (5 % wt-GFP and null-GFP) cells were allowed to develop on non-nutrient, buffered 1 % agar. Images were captured at aggregation (*A*, top panel), at the mound/tipped-mound stage (*A*, middle panel) and slug stages of development (*A*, bottom panel). Wt-GFP and null-GFP cells were uniformly distributed in predominantly wt and null structures, respectively. Wt-GFP cells sorted to the tips of predominantly null mounds and slugs, while null-GFP cells were excluded from the tips of predominantly wt mounds and sorted to the rear of predominantly wt slugs. Time-lapse video microscopy of mounds consisting of predominantly wt cells, mixed with 1–2 % of wt-GFP or null-GFP cells, were imaged and the paths of GFP expressing cells tracked (*B*). The open symbols represent the start point of the cells tracked and each subsequent symbol represents movement of the cell after 30 s. The localisation of wt-GFP or null-GFP cells within predominantly wt migrating slugs, with respect to Neutral Red staining, are shown (*C*). The darker stained portion of slugs (dashed line) indicates the portion of the slug that is stained with Neutral Red, a stain used to demarcate the pre-stalk region of slugs.

the null-GFP cells tended to move in a less circular manner and in a few instances, moved towards the periphery of the mounds. This apart, the speed and distance moved by the wt-GFP and null-GFP cells, was similar. Thus, the difference in the movement between wt and null cells may likely be enough to allow wt cells to sort preferentially to the tip over null-GFP cells.

At the slug stage of multi-cellular development, within $wt^{95\%}$/$wt\text{-}GFP^{5\%}$ mixtures, wt-GFP cells were uniformly distributed. In slugs consisting of $wt^{95\%}$/$null\text{-}GFP^{5\%}$, the null-GFP cells were excluded from the tip and instead accumulated towards the rear of slugs (Fig. 5C). To determine more precisely to where within those slugs the null-GFP cells sorted, $wt^{95\%}$/$null\text{-}GFP^{5\%}$ cell mixtures were stained with Neutral Red, before being allowed to develop (Fig. 5C) [15]. Neutral Red is used to stain pre-stalk cells that make up 20% of the cells within multi-cellular structures and sort to the tip of mounds and remain within the front fifth of migrating slugs (Fig. 5C). Neutral Red is indicated by the darker portion of slugs as viewed using phase microscopy. $Wt^{95\%}$/$wt\text{-}GFP^{5\%}$ slugs show darker staining at their front fifth portions, while the distribution of wt-GFP cells is uniform. Similarly, with $wt^{95\%}$/$null\text{-}GFP^{5\%}$, slugs were observed Neutral Red staining at the front fifth of slugs but the distribution of null-GFP cells was towards the rear end of slugs.

Mixtures of $null^{95\%}$/$null\text{-}GFP^{5\%}$ rarely formed slugs but the null-GFP cells were uniformly distributed throughout the structures formed (Fig. 5A). Although mixtures of $null^{95\%}$/$wt\text{-}GFP^{5\%}$ cells also rarely formed slugs, the wt-GFP cells did accumulate at the tips of mounds and, where a slug did form, wt-GFP cells remained within tips (Fig. 5A). Thus, null cells are reluctant in forming the organising centre known as the tip and are unable to fully participate in multi-cellular development within a predominantly wt background.

Increasing the amount of wt-GFP cells within null cells can partially rescue slug migration. Whereas within wholly wt mounds the tip would elongate from the mound and lead the cells within the mound to follow, in the case of mounds consisting of $null^{95\%}$/$wt\text{-}GFP^{5\%}$ cells, the mound failed to follow the wt-GFP cells within the tip (Fig. 6A, arrowheads). Instead, the tip eventually retracted and fell back into the mound. This processes occurred a number of times, after which a terminal structure was eventually formed.

Increasing the ratio of wt-GFP cells to the null cells, from 5% to 10% ($null^{90\%}$/$wt\text{-}GFP^{10\%}$ cells), led to a partial rescue in the tip-induced elongation of the mound (Fig. 6B, Supplemental Movie 6). In most cases, the tip, consisting primarily of wt-GFP cells, induced elongation of the mound and led to the formation of a slug capable of migrating, though not like wt slugs. Whereas wt slugs moved in a smooth motion, $null^{90\%}$/$wt\text{-}GFP^{10\%}$ slugs migrated in a jerky motion and in many cases seemed to drag a bulge at their rear ends (Fig. 6B, white asterisks). Taken together these data suggest that null cells form the organising centre known as the tip reluctantly and they are also unable to respond properly to signals from the tip. Thus, movement as a cohesive tissue is impaired.

Discussion

Previously we have shown that expression of FrmA is increased upon starvation, while FrmA null (null) cells have increased cell-substrate adhesion, making non-starved null cells and null cells in the early stages of starvation defective in cell migration [10]. The data presented here shows that FrmA expression continues to increase throughout multi-cellular development, while null cells have increased cell-cell adhesion and impaired multi-cellular development. In particular, FrmA null cells are excluded from the organising centre known as the tip, suggesting a role for FrmA in tip formation.

Although a number of cell-cell adhesion systems have been identified in *Dictyostelium* cells, mediated by CadA, CsA or LagC, respectively, their regulation and how they function with respect to each other is poorly understood. The increased cell-cell adhesion of null cells cannot be explained by an increase in expression of those cell-cell adhesion molecules. However, an increase in uncharacterised molecules, similar to CadA (DDB0191303) or LagC (DDB0216860, DDB0238302 and DDB0201624) present in the *Dictyostelium* sequencing database, cannot be ruled out. The largest difference in the expression of adhesion molecules between wt and null cells is actually in the greatly delayed onset of LagC expression in the null cells. LagC is a non-diffusible cell surface molecule that regulates cell-cell adhesion in an EDTA-insensitive manner. It is highly expressed during mound formation, while its deletion leads to mound arrest due to an inability to establish the organising centre known as the tip [7, 14]. The reason for the delayed expression of LagC is unclear but is not without precedent, as loss of LagD also leads to the greatly delayed and reduced expression of LagC [13]. LagD is similar in sequence and structure to LagC, and multi-cellular development of LagD null cells is even more severely impaired than in LagC null cells. Whereas LagC null cells arrest at the mound stage, LagD nulls cells fail to form mounds at all. Moreover, over-

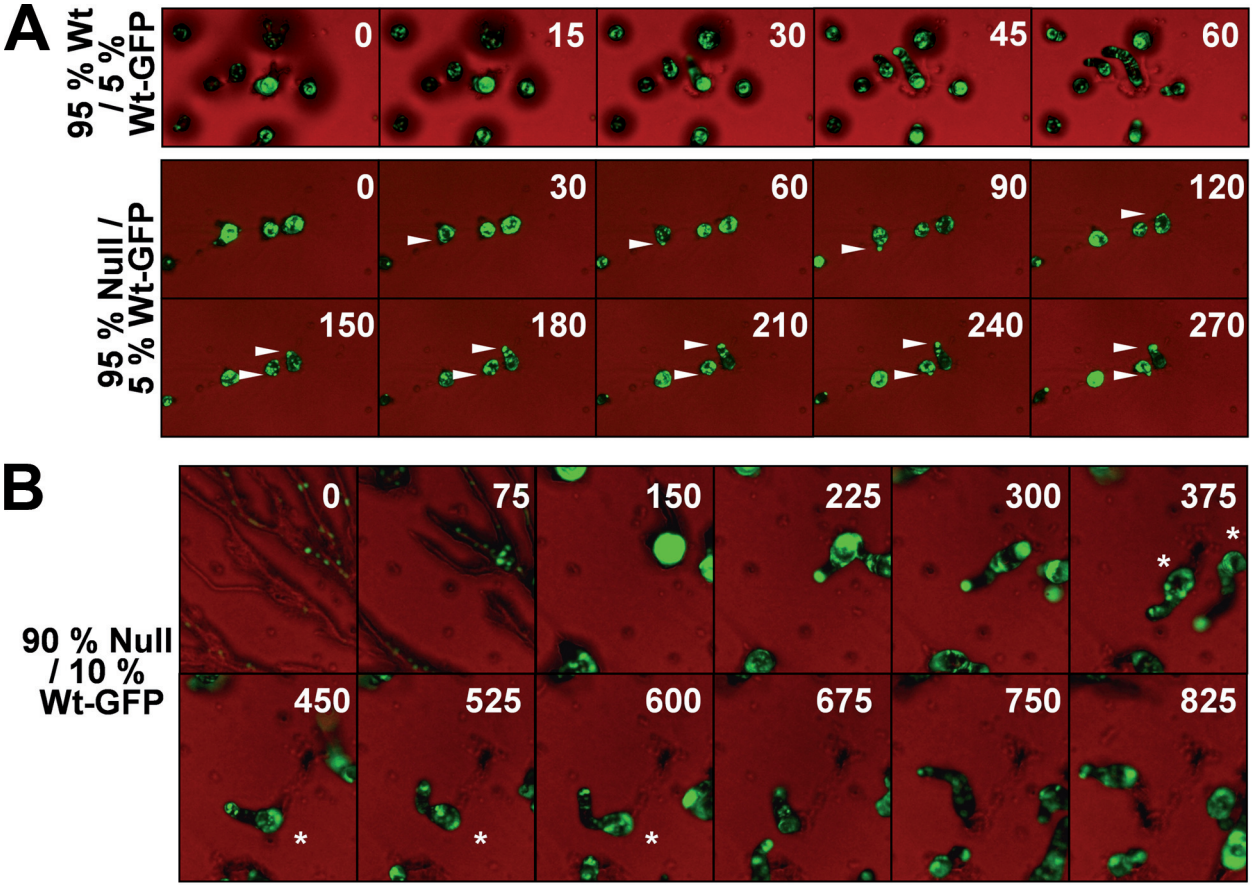

Figure 6. Increasing the number of wt-GFP cells in mixtures of null and wt-GFP cells partially rescues slug formation and migration. Whereas mixtures consisting of 95 % null cells and 5 % wt-GFP cells rarely form migrating slugs and often formed tips that underwent rounds of formation and retraction (*A*, arrowheads), mixtures consisting of 90 % null cells and 10 % wt-GFP cells are able to form migrating slugs (*B*, asterisks).

expression of LagC in either LagC null or wt cells, results in a delay at the mound stage of multi-cellular development. This suggests two things; first that the mere presence of LagC is not enough for wt multi-cellular development but rather a precise amount is required and, second, that tip formation requires very precise regulation of cell-cell adhesion. Thus, the reduced levels of LagC seen in the FrmA null cells may indicate a substantial deregulation of cell-cell adhesion within FrmA null cells and lead to their inability to efficiently form a tip. Data presented here suggests a link between FrmA and the regulation of LagC expression during multi-cellular development and may also explain their similar phenotypes.

Apart form LagC, which regulates cell-cell adhesion, deletion of molecules that regulate cell-substrate adhesion also result in impaired multi-cellular development in *Dictyostelium*. Loss of either paxillin or talinB results in impeded mound to slug transition and mound arrest, respectively [16, 17]. Indeed the phenotypes of, paxillin, talinB, LagC and FrmA null cells are all quite similar. TalinB and LagC null cells arrest at the mound stage and both are unable to form tips, while paxillin and FrmA null cells form mounds and only seem to go on to form tips reluctantly. Although paxillin and FrmA null cells progressed to the slug stage reluctantly, the slugs that were produced did not migrate. Their reluctance to form tips and the lack of slug migration are possible indicators of an inability to move as a cohesive tissue. Further, talinB is thought to be required for cells to move within a multi-cellular environment (*i.e.* within a tissue) while its FERM domain shares highest identity to the FERM domains of FrmA, suggesting a possible functional link between the two molecules [18]. Thus, to move as a cohesive tissue, both cell-cell and cell-substrate adhesion are required to be regulated precisely. In summary, paxillin, talinB and LagC all seem to be linked to FrmA. First, FrmA null cells show impaired localisation dynamics of paxillin [10]. Second, the talinB FERM domain is highly similar to the FERM domains of FrmA and the phenotypes of FrmA nulls and talinB nulls are also similar. Third, FrmA null cells show delayed expression of LagC. Thus, FrmA may represent a point of regulation that links cell-cell and cell-substrate

adhesion during multi-cellular development of *Dictyostelium.*

Electronic supplementary material. Supplementary material is available in the online version of this article at springerlink.com (DOI 10.1007/s00018-008-8527-y) and is accessible for authorized users.

Acknowledgements. This research was funded by the AICR.

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