Nucleocytoplasmic shuttling mediates the dynamic maintenance of nuclear Dorsal levels during Drosophila embryogenesis

Robert DeLotto1,*, Yvonne DeLotto1, Ruth Steward2 and Jennifer Lippincott-Schwartz3

In Drosophila, the NF-κB/REL family transcription factor, Dorsal, redistributes from the cytoplasm to nuclei, forming a concentration gradient across the dorsoventral axis of the embryo. Using live imaging techniques in conjunction with embryos expressing a chimeric Dorsal-GFP, we demonstrate that the redistribution of Dorsal from cytoplasm to nucleus is an extremely dynamic process. Nuclear Dorsal concentration changes continuously over time in all nuclei during interphase. While Dorsal appears to be nuclearly localized primarily in ventral nuclei, it is actively shuttling into and out of all nuclei, including nuclei on the dorsal side. Nuclear export is blocked by leptomycin B, a potent inhibitor of Exportin 1 (CRM1)-mediated nuclear export. We have developed a novel in vivo assay revealing the presence of a functional leucine-rich nuclear export signal within the carboxyterminal 44 amino acids of Dorsal. We also find that diffusion of Dorsal is partially constrained to cytoplasmic islands surrounding individual syncitial nuclei. A model is proposed in which the generation and maintenance of the Dorsal gradient is a consequence of an active process involving both restricted long-range diffusion and the balancing of nuclear import with nuclear export.

KEY WORDS: NF-κB, Gradient formation, Transcription factor dynamics, Drosophila

INTRODUCTION
The transcription factor NF-κB plays a pivotal regulatory role in an ever increasing number of important biological processes, including adaptive and innate immunity, apoptosis, inflammation, cell fate determination during development and cancer (Li and Stark, 2002). In the absence of active signaling, NF-κB is typically found in the cytoplasm in a 2:1 complex with its binding partner I-κB. The paradigm for NF-κB nuclear translocation argues that signal-dependent phosphorylation, ubiquitination and proteasome-mediated degradation of I-κB frees NF-κB from cytoplasmic anchoring, allowing it to translocate to the nucleus (Baeuerle and Baltimore, 1988; Baeuerle and Baltimore, 1989; Ghosh and Baltimore, 1990). There it can bind to κB binding sites and either upregulate or downregulate appropriate sets of target genes (Pierce et al., 1988). As proteolytic degradation of I-κB is thought to be the rate-limiting event determining nuclear uptake, one would expect that NF-κB nuclear translocation should be a relatively irreversible process. Indeed, most of the published biochemical and immunohistochemical data have been interpreted in accordance with this conventional view (Rothwarf and Karin, 1999). However, more recently, aspects of the paradigm are being re-evaluated (for a review, see Ghosh and Karin, 2002).

A classical model system mediated by NF-κB is dorsoventral (DV) patterning in the early D. melanogaster embryo (Moussian and Roth, 2005). In syncytial blastoderm embryos, in response to an extracellular signal, the NF-κB transcriptional regulator, Dorsal, forms a ventral-to-dorsal concentration gradient, with high levels in ventral nuclei and progressively lower levels in more dorsolateral nuclei (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989; Steward et al., 1988). Globally, nuclear Dorsal concentrations determine dorsal/ventral cell fates by either upregulating or downregulating the transcription of the zygotic target genes twist, snail, rhomboid and zerknullt, thus specifying relative DV position (Stathopoulos and Levine, 2002). The graded distribution of Dorsal among nuclei is most readily observed at nuclear cycle 14, during the period of time in which cellularization occurs. However, Dorsal already functions as a transcriptional regulator earlier at the syncytial blastoderm stage. For example, Dorsal transcriptionally regulates the expression of snail at nuclear cycle 11 and later (Alberga et al., 1991). Immunohistochemical staining shows that as early as nuclear cycle 10, when nuclei arrive at the surface of the embryo, Dorsal is distributed in a characteristic gradient, with high concentrations in ventral nuclei and an apparent absence from dorsal nuclei (Roth et al., 1989; Steward, 1989). From nuclear cycle 10 to 14, syncytial blastoderm nuclei undergo four complete mitotic divisions within a shared cytoplasm before cellularization occurs (Foe and Alberts, 1983).

We wished to study the dynamics of Dorsal redistribution and gradient formation within the early embryo. To do so, we utilized real-time live imaging of embryos expressing a fully functional form of Dorsal fused to the green fluorescent protein (GFP). We find that nuclear Dorsal concentrations are almost constantly changing and that the gradient completely breaks down and reforms with each mitotic division. Before reappearance of the gradient, we observe a transient accumulation of Dorsal in particles near the ventral, but not the dorsal, plasma membrane. Surprisingly, during interphase, when the Dorsal gradient appears to be relatively stable, Dorsal is dynamically shuttling into and out of nuclei. We demonstrate that Dorsal nuclear export is blocked by leptomycin B (LMB), a potent inhibitor of CRM1-mediated nuclear export and identify a region containing a leucine-rich nuclear export signal (LRNES). Lastly, we show that Dorsal is not distributed uniformly within the cytoplasm as previously thought from antibody stainings of fixed embryos but instead is partially compartmentalized into cytoplasmic domains.

1Department of Molecular Biology, University of Copenhagen, Ole Maaløes Vej 5, DK-2200 Copenhagen N, Denmark. 2Department of Molecular Biology and Biochemistry, Rutgers University, Waksman Institute, 190 Frelinghuysen Rd, Piscataway, NJ 08854-8020, USA. 3Cell Biology and Metabolism Branch, NICHD, NIH, Bldg. 1, 8T, 18 Library Drive, Bethesda, MD 20892, USA.

*Author for correspondence (e-mail: rdelotto@my.molbio.ku.dk)

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associated with individual syncitial nuclei. We propose a model for Dorsal redistribution between the cytoplasm and nucleus in which the redistribution of Dorsal is an active process and the function of the ventralizing signal is to alter the balance between nuclear import and nuclear export.

MATERIALS AND METHODS

Embryos

The Dorsal-GFP transgenic line was generated utilizing a strategy employed previously for Dorsal-β-galactosidase fusion (Drier et al., 1999), substituting GFP for the β-galactosidase cassette. A third chromosomal transgene was crossed into dl8/dl8, an amorphic allele fully rescuing the viability of embryos produced by homozygous females. Embryos were collected and dechorionized as described (DeLotto, 2001). Embryos were transferred to siliconized chambers (Lab-Tek), covered with Drosophila Ringer’s solution and oriented for imaging with an eyelash. We find that the interaction between the vitelline membrane and the siliconized surface allows the embryos to stick to the coverglass surface, enabling the positioning at a desired orientation.

Microscopy and imaging

Confocal microscopy was conducted on a Zeiss 510 Confocor 2 microscope using the 488 nm Argon laser line with a Zeiss 63× and 40× C-Apochromat water immersion objectives as previously described (Frescas et al., 2006). Data for Fig. 3 were quantified on Zeiss Confocor microscopy software release 2.8, while data for Fig. 1C were quantified using NIH Image. Files were converted to TIFF format and Quicktime movies generated using Adobe ImageReady 7.0. The high-resolution Quicktime movies are available at http://www.imbf.ku.dk/DeLotto_Lab/.

Cell culture and nuclear export assays

For leptomycin B treatment, two protocols were used interchangeably. Embryos were dechorionized, permeabilized by rinsing in Isopropanol for 3 seconds and Hexane for 5 seconds. Residual heptane was allowed to evaporate off and embryos were immediately immersed in phosphate-buffered saline (PBS). Under these conditions development proceeded normally for at least 4 hours in PBS. Leptomycin B (LMB) (a kind gift of Mary Dasso, NIH, Bethesda, MD) was added to the PBS at a final concentration of 10 μg/ml by dilution from a 10 mg/ml stock in Ethanol. Alternatively LMB was microinjected as a 1 mg/ml stock in 90% DMSO/10% ethanol. Anti-Twist stainings were conducted as previously described (Smith and DeLotto, 1994).

In vivo nuclear export assays were conducted as follows. Tandem GFP constructs were made by digesting pUASP with NotI and Xhol and inserting the NotI + Xhol-digested product of a PCR reaction using DG1, AGTCATATAAGCGGCGCGCTAACCACCATGTAGAAGCAAGGGCAG GAG; DG2, GATCCATAGCGAATTCTCCACCACCTCCCTTGTACAG CTGCTGATCCGCC; DG3, CAGTCGCTGGAATTCAATAATGGCGCA GAG; DG2, GATCCATAGCGAATTCTCCACCACCTCCCTTGTACAG CTGCTGATCCGCC; DG4, GTACACGCTCTCAGATTACGTGTGATATG GACAGTGTTCATCTGCAGATCTCCGAAATGGACGGCGACG; and DG7, GTACAGCTCTCAGATTACGTCGTGATATGACAGTGTTCATCTGCAGATCTCCGAAATGGACGGCGACG. Two GFP constructs were made by digesting pUASP with NotI and Xhol and inserting the NotI + Xhol-digested product of a PCR reaction using DG1 and DG7 on pMTNES+ (a gift of Janny L. Sørensen, Department of Molecular Biology, University of Copenhagen, Denmark) DG1 and DG2 were used in PCR with pMTNES+ to generate a dual GFP fragment, which was digested with NotI and EcoRI. DG3 and DG4 were used in PCR with embryonic dorsal CDNA and cut with EcoRI and Xhol. The two fragments were ligated into NotI + Xhol-digested pUASP (Roth, 1998). w1118 flies were transformed by P-element-mediated germline transformation using standard methods (Rubin and Spradling, 1982).

RESULTS

The Dorsal gradient breaks down before, and reforms after, mitosis

To study the fate of the Dorsal gradient through successive nuclear mitoses, we imaged living transgenic Drosophila embryos expressing Dorsal-GFP. The embryos used in these studies are

![Fig. 1. Breakdown during mitosis and reformation of the Dorsal gradient during interphase.](image-url)
idea that the gradient is generated by cycles of redistribution of Dorsal between the nuclear and cytoplasmic compartments. The timing of the loss of Dorsal from nuclei just before mitosis suggested that the integrity of the nuclear envelope is essential to maintenance of differential nuclear concentrations, as nuclear envelopes are known to become leaky at the beginning of mitosis due to nuclear pore breakdown (Kiseleva et al., 2001).

To better understand how Dorsal redistributes through successive nuclear cycles, we quantified the intensity of Dorsal-GFP within a single nucleus and one of its daughters at the same relative ventral position over time (Fig. 1C). As the nucleus entered interphase, nuclear fluorescence increased rapidly at first and then slowly throughout interphase in a ‘shark-tooth’ pattern. While the nuclear concentration increased continuously during syncitial interphases, it was lost abruptly at the start of mitosis. Thus, with the exception of nuclear cycle 14, nuclear Dorsal levels are highly variable and undergo continuous change during interphase.

**Dorsal redistributes between cytoplasmic, plasma membrane and nuclear pools during nuclear division cycles**

To examine local changes in the Dorsal distribution surrounding nuclei, we imaged a lateral section of the embryo at a zone of intermediate nuclear Dorsal concentration. As shown in Fig. 2A, during interphase, Dorsal-GFP was depleted from dorsolateral nuclei and enriched in ventrolateral nuclei, with a middle transition zone where nuclear levels match cytoplasmic levels (Fig. 2A, interphase). Loss of the gradient occurred in two stages. First, Dorsal-GFP levels dropped in ventral nuclei and levels increased in dorsal nuclei, giving rise to an equivalent, low level of Dorsal within all nuclei (Fig. 2A, early prophase). Then, Dorsal-GFP equilibrated between the dividing nuclei and the cytoplasm, resulting in a near uniform distribution late in mitosis (Fig. 2B, late mitosis). Because the level of nuclear fluorescence in both dorsal and ventral nuclei became equivalent at the start of mitosis and lowered in dorsal nuclei during interphase, the data suggested that during interphase an export mechanism might be necessary to reduce nuclear Dorsal levels in dorsal nuclei to levels below that of the surrounding cytoplasm.

To study the reappearance of the gradient, we viewed the cytoplasm just beneath the plasma membrane on the ventral side of the embryo. The diffuse cytoplasmic distribution of Dorsal-GFP observed in mitosis (Fig. 2B, late mitosis) changed as soon as cells entered interphase. In ventral nuclei, Dorsal-GFP first accumulated throughout the nucleoplasm (Fig. 2B, mid-interphase), but later could be seen highlighting condensed chromosomes (Fig. 2D). In control experiments in which recombinant tetrameric dsRED was microinjected into embryos, dsRED was excluded from the condensed chromosomes (data not shown). This suggests a form of interaction between Dorsal and the chromatin. More interestingly, before reappearing in ventral nuclei, Dorsal-GFP was transiently observed in a particulate compartment at or near the plasma membrane (Fig. 2B, see arrow), which in a confocal surface view could be seen at the hexagonal borders of cytoplasmic domains defined by dividing nuclei (Fig. 2C, VENTRAL). Significantly, this particulate distribution was observed only on ventral and ventrolateral plasma membrane, where substantial levels of Dorsal accumulate in nuclei, and was never observed on the dorsal side of the embryo (Fig. 2C, DORSAL). Given that Dorsal has been demonstrated to form a biochemical complex with Cactus, Tube and Pelle, and this complex is likely to be recruited to activated Toll receptors present only on the ventral and ventrolateral plasma membrane (Towb et al., 1998; Yang and Steward, 1997), the particulate distribution of Dorsal-GFP could correspond to Dorsal transiently binding to activated Toll receptor complexes.

**Dorsal is highly mobile within nuclei and is shuttling into and out of syncitial nuclei throughout the embryo**

We performed quantitative photobleaching experiments to study the binding, diffusion and transport properties of Dorsal during syncytial and cellular blastoderm stages. Photobleaching of a small spot...
considerably less than the diameter of a ventral nucleus for 3 seconds, caused all nuclear Dorsal-GFP fluorescence to drop to cytoplasmic background level (see Fig. 3A,B). This indicated that most of the Dorsal protein within nuclei is not avidly bound to DNA, as a pool of unbleached molecules outside the bleached spot would otherwise have been observed. The highly structured appearance of Dorsal-GFP observed in high resolution images of ventral nuclei (Fig. 2D), therefore, represents a dynamic state of Dorsal binding to and dissociating from chromatin, as has been reported for other nuclear proteins (Handwerger et al., 2003; Phair and Misteli, 2001).

We next monitored fluorescence recovery (FRAP) to determine whether the nuclear Dorsal pool was in exchange with the cytoplasmic pool. Over time, we observed that nuclear fluorescence recovered much more rapidly than could be accounted for due to the rising ‘shark-tooth’ pattern of Dorsal increase previously described (see Fig. 3A). We quantified the fluorescence in the bleached nucleus and normalized it to another nucleus at the same relative DV position. This allowed us to subtract the contribution of the continuous increase described in Fig. 1C from our recovery kinetics. After normalization, 90% of the initial Dorsal-GFP fluorescence within the nucleus recovered within 180 seconds (Fig. 3A,C). This reveals that Dorsal undergoes rapid exchange between the nucleus and the surrounding cytoplasm. The data do not support the purely unidirectional nuclear import model, but instead suggests that both nuclear import and export simultaneously contribute to the generation of nuclear Dorsal concentrations during interphase.

Based upon numerous antibody stainings of fixed embryos, it has been generally argued that Dorsal is excluded from nuclei on the dorsal side of the embryo (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). To test whether Dorsal is excluded from dorsal nuclei, we performed photobleaching experiments. If Dorsal is present within dorsal nuclei, fluorescence intensity within the nucleus would be expected to be measurably reduced after photobleaching. As shown in Fig. 3B, upon photobleaching a small spot within a nucleus on the extreme dorsal side, the fluorescence intensity of Dorsal-GFP within the nucleus was reduced, indicating that Dorsal is present within dorsal nuclei. Over time, normalized fluorescence in the bleached nucleus recovered to about 80% of the level observed in surrounding nuclei before entry into mitosis (Fig. 3D). This indicated that Dorsal was also shuttling into and out of dorsal nuclei, as we have previously shown for ventral nuclei. Similar photobleachings of many ventral (n=14), dorsal (n=12) and lateral (n=6) nuclei revealed that Dorsal shuttles into and out of nuclei at all positions within the embryo (data not shown).

To confirm that Dorsal is being exported from nuclei, we selectively inhibited nuclear export using LMB (Fukuda et al., 1997). Embryos at the beginning of nuclear cycle 14 were treated with LMB and Dorsal-GFP was imaged up until gastrulation. During cellularization in untreated control embryos, Dorsal-GFP fluorescence never accumulated to high levels within dorsal and dorsolateral nuclei (Fig. 4A). By contrast, upon treatment with LMB, fluorescence dramatically increased in dorsal and lateral nuclei (Fig. 4B), and persisted well into the start of gastrulation (Fig. 4D), a time when wild-
type Dorsal protein begins to disappear (Fig. 4C). These results provide independent evidence for nucleocytoplasmic shuttling and indicate that the export process is CRM1-mediated.

To determine whether the accumulation of Dorsal within dorsal and lateral nuclei leads to ventralization, we introduced LMB into permeabilized wild-type embryos at the start of cellularization, fixed them at the end of cellularization and stained them with antisera against the mesodermal marker, Twist. Twist is normally expressed in 16 to 18 cells along the ventral midline of regions corresponding to very high levels of nuclear Dorsal (Leptin, 1991). As shown in Fig. 4E, treatment with LMB leads to expansion of Twist expression to cells on the dorsolateral side of the embryo, consistent with ventralization of the embryo. Evaluation of the later effects of LMB by standard cuticle preparations was not possible due to pleiotrophic ventralization of the embryo. Evaluation of the later effects of LMB provided independent evidence for nucleocytoplasmic shuttling and ventralization of the embryo.

**Dorsal contains a functional LRNES near its carboxyterminus**

As CRM1-mediated nuclear export is conducted via an LRNES, we examined the amino acid sequence of Dorsal for matches to the LRNES consensus (la Cour et al., 2004). We found several potential LRNESs clustered between amino acids 644 and 678 near the carboxyterminus. Because of the loose consensus for LRNESs, a number of alignments were possible. In Fig. 4F, we have illustrated two of several possible alignments within this region. To determine whether this region contains a functional nuclear export signal, we developed a native in vivo assay. We constructed P-element-mediated constructs used in an in vivo assay for nuclear export. (H) Localization of Tandem GFP driven by nanos-GAL4. (I) Localization of tandem GFP with Dorsal's carboxyterminal 44 amino acids driven by nanos-GAL4. (J) Alignment of Putative LRNES sequences at the carboxyterminus with other Drosophila Nf-kB proteins and several mammalian LRNESs.

**Dorsal does not freely diffuse throughout the cytoplasm but partitions into cytoplasmic domains surrounding individual syncitial nuclei**

It has been commonly argued that Dorsal is distributed uniformly within the cytoplasm of the syncytial and blastoderm embryo (Roth et al., 1989; Rushlow et al., 1989; Steward et al., 1988). Our observation of nucleocytoplasmic shuttling prompted us to address the question of how freely Dorsal moves within the cytoplasm. Dorsal does not freely diffuse within the cytoplasm, because of nucleocytoplasmic shuttling, repetitive photobleaching of a small spot in the cytoplasm should cause neighboring nuclei to be relatively equally reduced in their nuclear fluorescence intensities. Surprisingly, when we performed FLIP (fluorescence loss in photobleaching) upon a small spot of ventral cytoplasm (Fig. 4H), the distribution mirrors that observed for microinjected recombinant GFP in embryos (data not shown). By contrast, 2×GFP-DLC also enters and is trapped within nuclei after mitosis; however, it is cleared from nuclei during interphase (Fig. 4I). This indicates that the carboxyterminal 44 amino acids of Dorsal are sufficient to mediate selective nuclear export in embryos during cellular blastoderm. A further characterization of the precise structure of the LRNES is currently underway.
fluorescence. This suggested that the cytoplasm surrounding individual nuclei may be partially compartmentalized with respect to the diffusion of Dorsal. We therefore more closely examined the dorsal side of the embryo and observed a structure to the distribution of Dorsal-GFP in regions surrounding each nucleus (Fig. 5B-D). While part of this structure may be trivially explained by infoldings of the plasma membrane, the pseudocleavage furrows, Dorsal-free areas often extended somewhat deeper than the pseudocleavage furrows, as shown by larger gaps (see Fig. 5B,C). To determine whether the diffusion of Dorsal is constrained by these domains, we photobleached a spot overlapping both the nucleus and the cytoplasm of one of these domains during mitosis (Fig. 5E). We observed that the cytoplasmic domain surrounding one nucleus was selectively darkened, indicating a form of cytoplasmic compartmentalization that persisted even throughout mitosis. However, as a substantial amount of the fluorescence rapidly recovered within 37.5 seconds, significant exchange between these domains and some other pool of Dorsal must also occur.

To determine whether these bleached domains recovered Dorsal from the deeper cytoplasm or from adjacent domains, we compared the lateral mobility of Dorsal within these two regions. To do this, we alternately photobleached (FLIP) a box from the plasma membrane to the deeper cytoplasm and imaged it, monitoring fluorescence loss in adjacent regions. As shown in Fig. 5F, at the surface relatively little fluorescence was lost outside of the bleach box (white) even after four bleaching cycles (5F, third panel), indicating that Dorsal is partially constrained in its mobility near the plasma membrane surface but is more freely diffusing in the lower, deep cytoplasm. Relative linear intensities are indicated by the color bar at the bottom left. (G) Dorsal is partially constrained in its diffusion to the part of the cytoplasm between the nucleus and the plasma membrane. A 5 second FRAP of nucleus (bleach box in red) transiently reduces fluorescence preferentially in the blue zone. In subsequent images (data not shown), cytoplasmic fluorescence is recovered with delayed kinetics by flow from the deep cytoplasm.
exchange does occur, albeit somewhat more slowly with a pool in the deeper cytoplasm. This restriction of Dorsal movement may be due to nuclear-associated cytoskeletal elements as well as a contribution from pseudocleavage furrows of the plasma membrane (Foe et al., 2000), as compartmentalization was locally lost when embryos were microinjected with the microtubule depolymerizing drug Nocodozole (data not shown).

**DISCUSSION**

Our data demonstrate that the redistribution of Dorsal from cytoplasm to nucleus is a more dynamic and complex process than previously thought. Before the completion of cellularization, the Dorsal gradient appears not once but five times, punctuated by mitotic divisions. Our observations are consistent with an early report suggesting that the Dorsal gradient broke down during mitoses (Roth et al., 1989). In order for this to occur, either the patterning system must generate the gradient de novo in a cyclical fashion, or it produces a ‘graded’ distribution of one component only once that component is relatively stable from nuclear cycle 10 through cellularization. A simple hypothesis is that once activated, Toll receptors are able to continuously signal and do not diffuse significantly within the plasma membrane. Continuous sensing of the local activation state of Toll receptors would permit the cytoplasmic part of the pathway to ‘read’ the ventralizing signal in real time. A requirement for Dorsal redistribution from cytoplasm to nucleus would be the integrity of nuclear pore complexes. As nuclear pore components fall off and nuclear pores become leaky with the start of mitosis, the Dorsal gradient breaks down, only to be re-established concomitant with the reassembly of nuclear pores at the start of interphase (Fuchs et al., 1983).

Before the reappearance of Dorsal within ventral nuclei, Dorsal transiently appears in a particulate distribution at the plasma membrane surface. This distribution is similar to that observed for the Toll receptor (Hashimoto et al., 1991) (and our unpublished observations). It has been previously demonstrated that Dorsal and Cactus form a biochemical complex with Toll (Yang and Steward, 1997). The transient appearance of Dorsal near the plasma membrane during syncitial and cellular blastoderm stages whenever a graded distribution is observed. In light of our data and the studies cited above, we propose a dynamic model for Dorsal nuclear redistribution (see Fig. 6). In the model, Dorsal bound to Cactus is recruited to a signaling complex at the plasma membrane by activated Toll receptors (Yang and Steward, 1997). There, both Dorsal and Cactus are phosphorylated (Drier et al., 1999). Phosphorylated Dorsal is released from the complex and enters the nucleus, where it moves between the nucleoplasm and binds nonspecifically to chromatin. Within the nucleus, Dorsal is dephosphorylated and then exported back to the cytoplasm by the CRM1-dependent nuclear export pathway. Once in the cytoplasm Dorsal associates with de novo synthesized Cactus, again forming a complex, repeating the cycle. Our model is similar to one that has been proposed for the SMADs, which mediate TGF-β signaling (Nicolas et al., 2004). In the case of the SMADs, a nuclear phosphatase has been demonstrated to exist; however, the specific enzyme has not yet been identified.

Our model departs from previous ones in that it places Dorsal in an active exchange between cytoplasm and nucleus. It also puts greater emphasis on phosphorylation of Dorsal with Cactus phosphorylation and degradation, providing a modulating effect upon the equilibrium. Interaction of Dorsal with Cactus might reduce the efficiency of Dorsal phosphorylation or locally slow the movement of Dorsal within the cytoplasm. In ventral regions of the cytoplasm more Cactus is phosphorylated and ultimately degraded, resulting in an inverse cytoplasmic gradient of Cactus (Bergmann et al., 1996; Reach et al., 1996).
The accumulation of Dorsal in dorsal nuclei upon LMB treatment indicates that CRM1-mediated nuclear export plays an important role in determining nuclear Dorsal concentration. It has been generally accepted that it is the nuclear import of Dorsal that is regulated (Morisato and Anderson, 1995). Our results raise the question of whether import alone, export alone or both import and export are regulated. The independent measurement of nuclear import and export rates will be necessary to answer this question. It will be interesting to determine whether some components of the pathway specifically alter the dynamic equilibrium via the export process. For example, although it has been argued that Tamo downregulates nuclear import, Tamo may alternatively function by increasing the nuclear export rate (Minakhina et al., 2003).

The partial compartmentalization of the cytoplasm surrounding each nucleus provides an interesting mechanism for isolation of the signaling environment between each individual nucleus within the common cytoplasm of the syncitial embryo. Dorsal protein would be expected to cycle primarily in the vicinity of each nucleus and only exchange more slowly with pools associated with neighboring nuclei. In this way, different nuclei could sample the ventralizing signal and maintain unique nuclear Dorsal levels independently of one another. This compartmentalization property might be common to components of other signalling pathways in the embryo and contribute to the transcriptional isolation of each nucleus, something that has been observed but for which no mechanism is known to exist.

Our results demonstrate that the redistribution of Dorsal from the cytoplasm to the nucleus is a dynamic process rather than a singular unidirectional event. While nucleocytoplasmic shuttling has been described for a number of transcription factors, our results extend the mechanism for maintenance of other signaling pathways, which crosstalk with the primary one. In the case of Dorsal, shuttling provides a mechanism for maintenance of the gradient through four mitotic divisions and may provide a mechanism for the terminal pathway to downregulate nuclear Dorsal levels in the anterior and posterior of the embryo (Rusch and Levine, 1994). Nucleocytoplasmic shuttling may be a general property of all NF-kB transcription factors, as our data are consistent with reports of nucleocytoplasmic shuttling of NF-kB in mammalian cells (Birbach et al., 2004; Birbach et al., 2002; Carlotti et al., 2000). In mammalian cells, nucleocytoplasmic shuttling could provide the ability to rapidly modulate nuclear NF-kB levels with fluctuations in cytokine levels. In Drosophila embryogenesis it may be central to the mechanism by which the Dorsal gradient is formed and maintained.

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