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

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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 proteosome-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

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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 insertion of this transgene was crossed into dl^8/dl^8, 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 generated using the following oligonucleotides: DG1, AGTCATATAAGCGGCCGCTAACCACCATGGTGAGCAAGGGCGAGGAG; DG2, GATCCATAGCGAATTCTCCACCACCTCCCTTGTACAGCTCGTCCATGCC; DG3, CGATCGCTGGAATTCAATAATGGGCCAACGCTCAGC; DG4, GTACAGCTCTCTAGATTACGTGGATATGGACAGGTTCGATATCTGCAGATCTTCCGAATTGAGGCGCAG; and DG7, GTACAGCTCTCTAGATTACTTGTACAGCTCGTCCATGCC. Two GFP constructs were made by digesting pUASP with *Not*I and *Xba*I and inserting the *Not*I + *Xba*I-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 *Not*I and *Eco*RI. DG3 and DG4 were used in PCR with embryonic *dorsal* cDNA and cut with *Eco*RI and *Xba*I. The two fragments were ligated into *Not*I + *Xba*I-digested pUASP (Rorth, 1998). w^{1118} 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

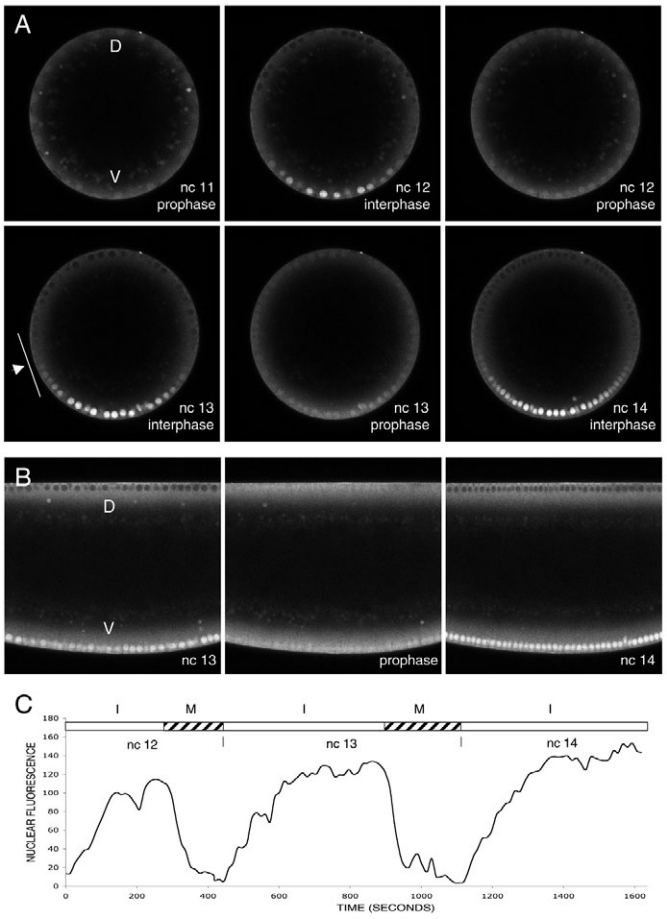

Fig. 1. Breakdown during mitosis and reformation of the Dorsal gradient during interphase. (**A**) Cross-sectional views of a live *Drosophila* embryo, showing the distribution of Dorsal-GFP from nuclear cycles 11 to 14; orientation is ventral down, dorsal up. (**B**) Saggital views, showing interphase nuclear cycle 13, mitosis (prophase) and interphase nuclear cycle 14; the orientation in all panels is ventral down, dorsal up. (**C**) A quantification of the relative nuclear fluorescence of one nucleus at a fixed position as a function of time from nuclear cycles 12 to 14. Nuclear fluorescence intensity was calculated as spot intensity within nuclei minus the spot intensity of the adjacent cytoplasm plotted against time in seconds. Relative periods of interphase and mitosis (shown by hatched bars) were determined by visualizing mitotic spindles that are readily apparent on the dorsal side via their transient interaction with Dorsal-GFP.

dl^8/dl^8 null alleles expressing a chimeric Dorsal-GFP fusion protein from the endogenous *dorsal* promoter fully rescuing the *dorsal* null phenotype with normal viability. Embryos viewed in cross-sectional orientation from nuclear cycle 11 through 14 revealed a gradient of Dorsal-GFP across interphase nuclei that was typical of endogenous Dorsal protein with dorsal nuclei depleted and ventral nuclei enriched in Dorsal (Fig. 1A, interphase) (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). Surprisingly, as nuclei entered mitotic prophase the gradient disappeared and reappeared at the end of mitosis when the next nuclear cycle began (Fig. 1A, prophase).

Wide-field fluorescence quantification over successive nuclear cycles indicated that the total Dorsal-GFP fluorescence in embryos did not dramatically change between interphase and mitosis (data not shown). This observation was inconsistent with the idea that the cyclic appearance of the gradient was caused by cycles of protein degradation and re-synthesis. Rather, it is more consistent with the

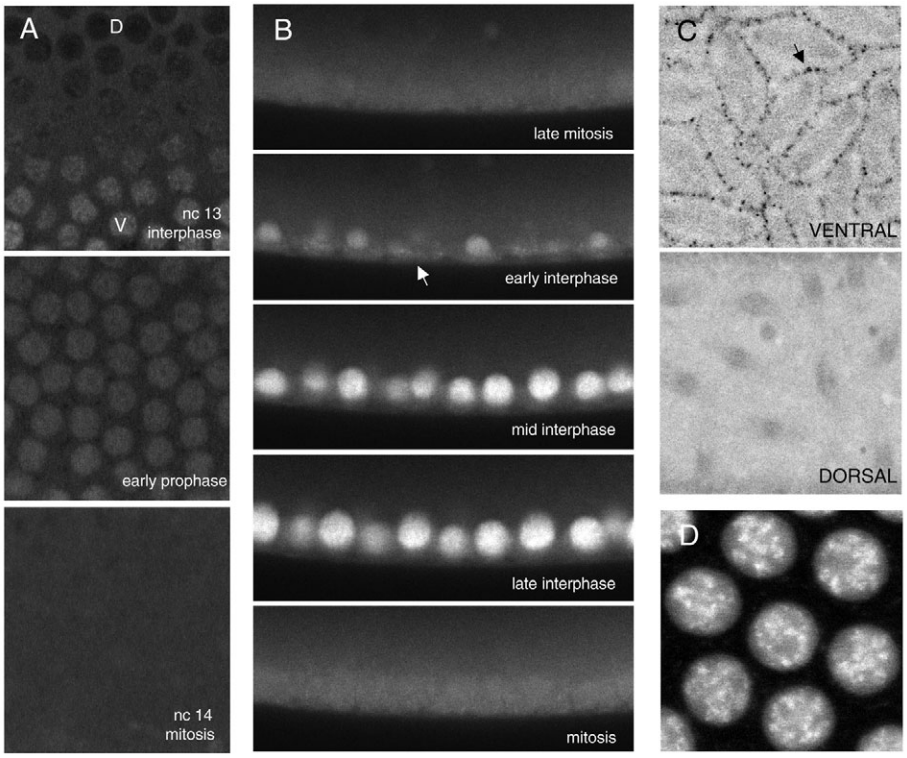

Fig. 2. Local redistribution of Dorsal as nuclei proceed through mitosis in *Drosophila*. (**A**) A lateral view of the transition between relative nuclear inclusion and exclusion (for orientation, see arrow in fourth panel of Fig. 1A). On the dorsolateral side, Dorsal transiently enters nuclei that previously excluded it at the start of mitosis (early prophase) and shortly afterward redistributes in a diffuse relatively uniform pattern (telophase, bottom panel). (**B**) A side view of the ventral surface showing a transient particulate distribution at the plasma membrane surface at the end of mitosis (white arrow). (**C**) A surface view (image inverted), showing the distribution of Dorsal-GFP in the particulate compartment on the ventral side (VENTRAL). Dorsal-GFP is never observed in a particulate compartment on dorsal plasma membranes, where nuclei do not take up Dorsal (DORSAL). (**D**) Dorsal is present within late ventral nuclei diffusely in the nucleoplasm and enriched in the chromosomal compartment.

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 distribution 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

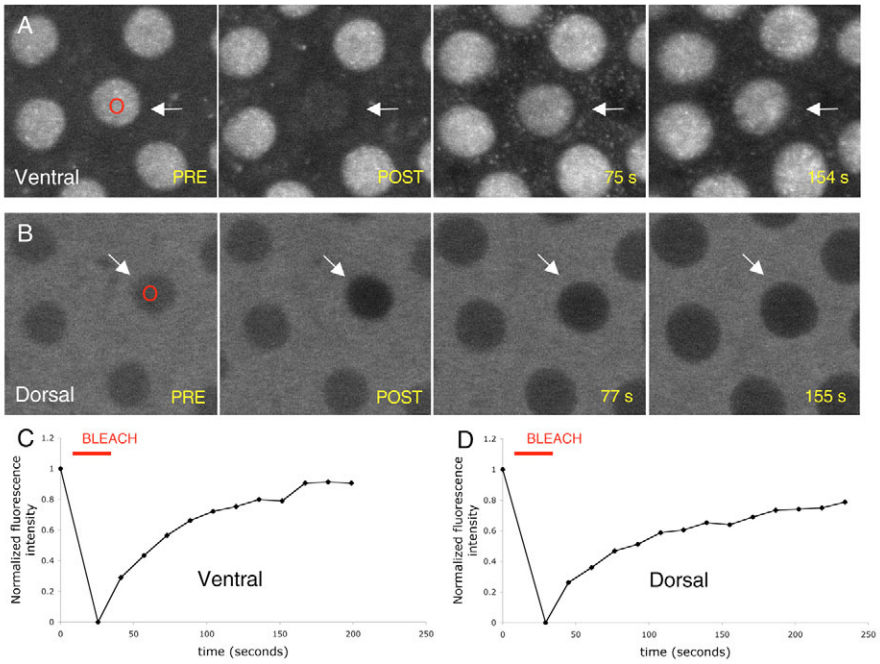

Fig. 3. In *Drosophila* Dorsal is highly mobile and shuttles from nucleus to cytoplasm in both ventral and dorsal nuclei. (**A**) A fluorescence recovery after photobleaching (FRAP) reveals that Dorsal-GFP is highly mobile within the nucleus. A bleached area (red circle) before and after a 3 second photobleach, showing extensive and uniform loss of nuclear fluorescence. This indicates high mobility of Dorsal-GFP within nuclei. However, the extensive fluorescence recovery by 154 seconds postbleach shows that nuclear Dorsal-GFP must exchange with the cytoplasmic pool and thus undergoes nucleocytoplasmic shuttling. (**B**) Dorsal is present in extreme dorsal nuclei and shuttles from nucleus to cytoplasm. An identical FRAP conducted on a nucleus located on the extreme dorsal side, showing both initial loss and subsequent recovery of nuclear fluorescence over time. (**C**) Quantification of the data from the ventral nuclear FRAP. To compesate for any change in nuclear levels over time, internal nuclear fluorescence intensity was normalized to that of a ventral nucleus at the same relative DV position. (**D**) Quantification of the data from the dorsal nuclear FRAP. As in C, internal nuclear fluorescence intensity normalized to a nearby (dorsal) nucleus at the same relative DV position. Bleach boxes in both experiments are shown in red.

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 for 3 seconds, 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-

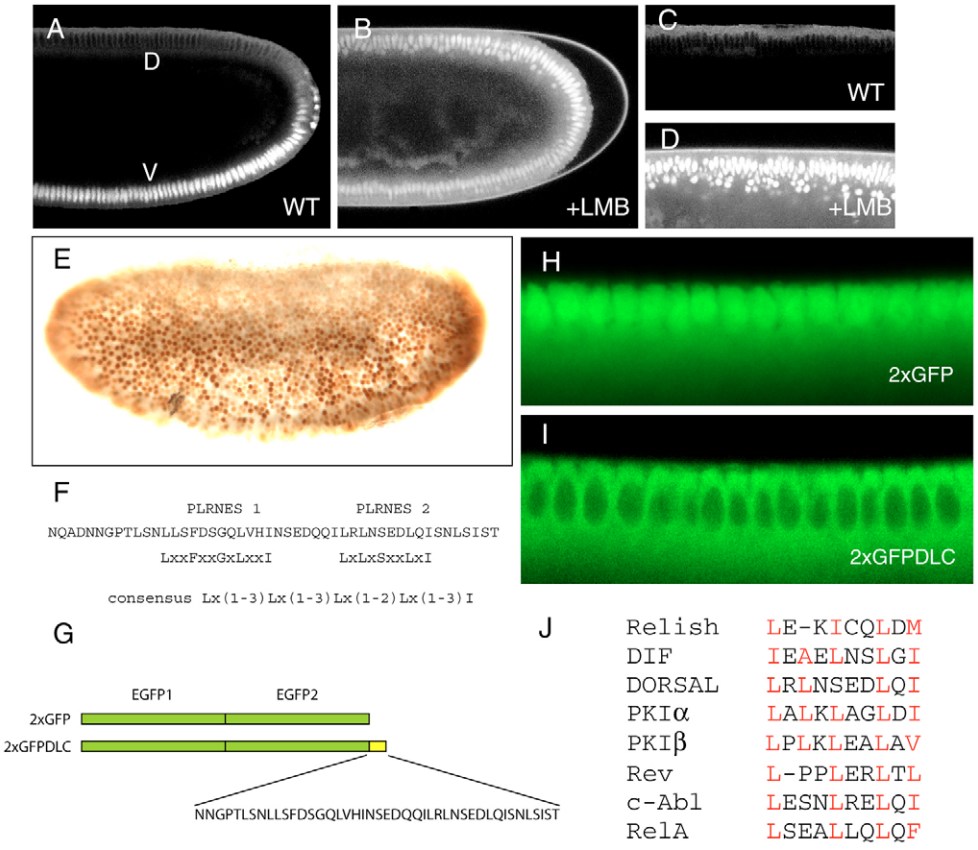

Fig. 4. Dorsal is exported from nuclei by a CRM1-mediated process and has a carboxyterminal LRNES. (**A**) A saggital confocal section of a wild-type *Drosophila* embryo, showing the normal polarized nuclear Dorsal distribution. (**B**) A saggital confocal section of an embryo treated with LMB during nuclear cycle 14, showing Dorsal protein within dorsal nuclei, which normally do not accumulate Dorsal. (**C**) A dorsal view of embryo at the beginning of gastrulation. (**D**) A dorsal view of an LMB-treated embryo, showing perdurance of high levels of nuclear Dorsal during gastrulation. (**E**) Anti-Twist staining of an LMB-treated embryo, showing expansion of mesodermal cell fates. (**F**) Putative LRNESs within the carboxyterminus of Dorsal. (**G**) Schematic of tandem GFP 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-κB proteins and several mammalian LRNESs.

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 at 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 effects of LMB treatment.

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 concensus 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 transformed fly lines expressing either tandem GFP (2×GFP) or tandem GFP with amino acids 635-678 of Dorsal (2×GFP-DLC) under the control of the GAL4 UAS (Fig. 4G). These transgenic lines were crossed to a nanos-GAL4 driver line and embryos were imaged to determine the localization of 2×GFP and 2×GFP-DLC. While the molecular weight of tandem GFP (58 kD) should not permit it to enter nuclei during interphase, it does enter nuclei and become trapped during each mitosis. In the absence of an export signal, 2×GFP remains within nuclei at levels slightly higher than in the surrounding 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.

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 syncitial 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 surrounding syncytial nuclei. If Dorsal diffuses freely 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. 5A), we found that several adjacent nuclei underwent only modest reductions in nuclear fluorescence, whereas one nearby nucleus showed a particularly dramatic reduction in

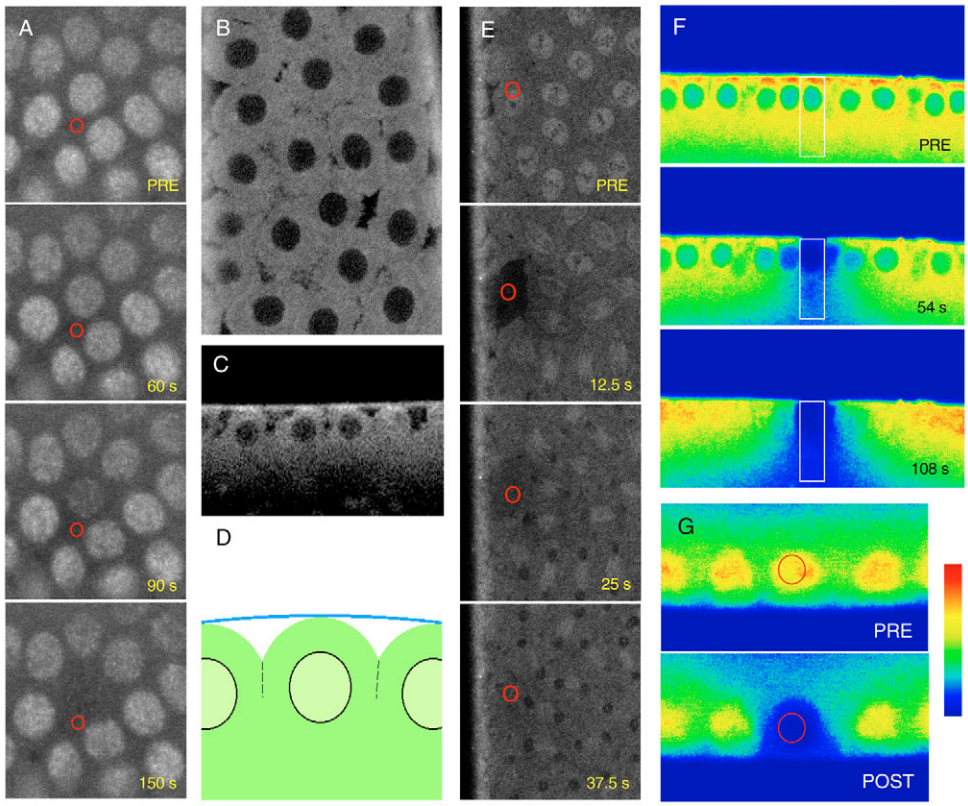

Fig. 5. Dorsal partitions into nucleus-associated cytoplasmic domains in *Drosophila*. (**A**) Photobleaching of the cytoplasm preferentially reduces nuclear Dorsal levels in one nearby nucleus. FLIP of the common cytoplasm results in the preferential reduction of nuclear fluorescence in one associated nucleus, suggesting compartmentalization of the syncitial cytoplasm. Bleach box is shown in red. (**B**) A dorsal surface view, showing high cytoplasmic levels of Dorsal adhering to nuclear-associated domains. (**C**) A side confocal view, showing dorsal nuclear domains. (**D**) A drawing of the domains. (**E**) A FRAP of both nucleus and cytoplasm on the ventral side at the beginning of mitosis that preferentially bleaches one cytoplasmic domain. Rapid recovery in the 25 and 37.5 second panels shows that Dorsal within the domain can exchange with a mobile pool from elsewhere within the embryo. (**F**) A false color image of an FLIP of peripheral and deep cytoplasm on the dorsal side (bleach box in white), 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.

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 lateral diffusion of Dorsal-GFP was highly limited near the plasma membrane. However, Dorsal-GFP fluorescence was substantially reduced in regions adjacent to the bleach box in the deeper cytoplasm, suggesting higher mobility of Dorsal within the deep cytoplasm. To examine the mobility within a domain and in the immediate proximity of the nucleus, we photobleached one ventral nucleus from the side and imaged the fluorescence immediately afterwards. As shown in Fig. 5G, a cup-shaped zone of cytoplasm is highly depleted within the part of the cytoplasm between nucleus and plasma membrane, while Dorsal-GFP appears to be re-entering the domain from the deep cytoplasm. These results are consistent with a form of partial compartmentalization in which Dorsal is relatively free to move within a zone between a single nucleus and its intimately associated cytoplasm but is more constrained in its movement laterally at the boundary between each adjacent domain. However,

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 might result from stalling at activated Toll receptor due to a delay in the re-establishment of functional nuclear pore complexes at the completion of mitosis.

Data from a number of published studies suggest that the regulation of Dorsal is a more complex process than release from cytoplasmic anchoring via its I-κB partner, Cactus. For example, Dorsal still differentially translocates from cytoplasm to nuclei and can form a gradient in embryos that are completely lacking its I-κB partner, Cactus (Bergmann et al., 1996; Reach et al., 1996; Roth et al., 1991). This result has been explained by postulating the existence of another, as yet unidentified, I-κB partner that partially complements Cactus function (Reach et al., 1996). An alternative explanation is that Cactus is not the sole determinant of Dorsal nuclear translocation and at least another parallel input is required. Several studies have shown that the phosphorylation of Dorsal is required for Dorsal to appear within the nucleus (Drier et al., 2000; Drier et al., 1999; Gillespie and Wasserman, 1994). Two parallel intracellular signals have been suggested to direct nuclear translocation; the first, Cactus phosphorylation and degradation, and the second, Dorsal phosphorylation. Interestingly, Dorsal phosphorylation is absolutely required for redistribution from cytoplasm to nucleus, while the Cactus-dependent part of the pathway is not essential to nuclear entry (Bergmann et al., 1996; Drier et al., 2000; Drier et al., 1999).

Our data reveal that Dorsal is in a dynamic equilibrium continuously shuttling between the cytoplasm and the nucleus 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).

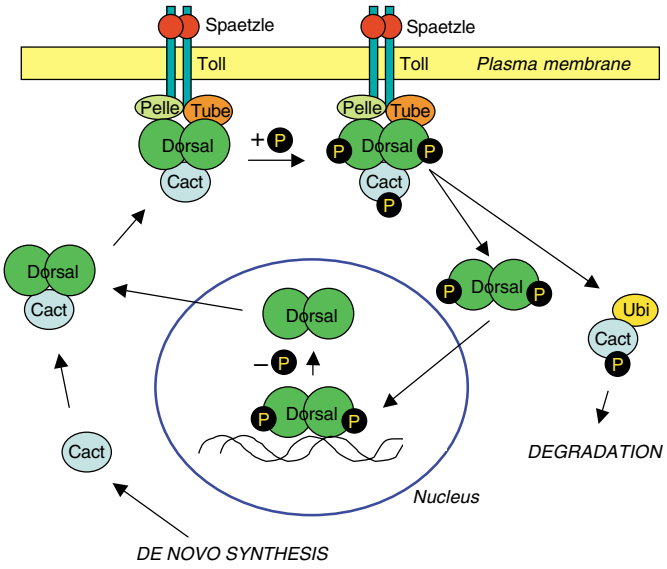

Fig. 6. A model for redistribution of Dorsal between cytoplasm and nucleus as a dynamic equilibrium between the activated Toll receptors and the nucleus in *Drosophila*. Dorsal and Cactus are recruited and phosphorylated by active Toll membrane receptor complexes on the ventral plasma membrane. Phosphorylated Cactus is degraded and phosphorylated Dorsal translocates to the nucleus. Within the nucleus it becomes dephosphorylated and is exported, re-entering the cytoplasmic pool binding de novo synthesized Cactus. Dorsal is again recruited to active receptor complexes and the cycle repeats itself. Partial compartmentalization of the cytoplasm assures integration of the signal from only local active signaling complexes and buffering from signaling complexes associated with the plasma membrane in close proximity to adjacent nuclei.

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 process to the formation of a developmental gradient during embryogenesis. Dynamic shuttling provides for exquisite control to the regulation of nuclear levels of transcription factors, allowing nuclei to rapidly adapt to changing levels of signal input or to integrate the effects 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-κB transcription factors, as our data are consistent with reports of nucleocytoplasmic shuttling of NF-κB 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 NF-κB 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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References

Alberga, A., Boulay, J. L., Kempe, E., Dennefeld, C. and Haenlin, M. (1991). The snail gene required for mesoderm formation in Drosophila is expressed dynamically in derivatives of all three germ layers. *Development* **111**, 983-992.

Baeuerle, P. A. and Baltimore, D. (1988). Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor. *Cell* **53**, 211-217.

Baeuerle, P. A. and Baltimore, D. (1989). A 65-kappaD subunit of active NF-kappaB is required for inhibition of NF-kappaB by I kappaB. *Genes Dev.* **3**, 1689-1698.

Bergmann, A., Stein, D., Geisler, R., Hagenmaier, S., Schmid, B., Fernandez, N., Schnell, B. and Nusslein-Volhard, C. (1996). A gradient of cytoplasmic Cactus degradation establishes the nuclear localization gradient of the dorsal morphogen in Drosophila. *Mech. Dev.* **60**, 109-123.

Birbach, A., Gold, P., Binder, B. R., Hofer, E., de Martin, R. and Schmid, J. A. (2002). Signaling molecules of the NF-kappa B pathway shuttle constitutively between cytoplasm and nucleus. *J. Biol. Chem.* **277**, 10842-10851.

Birbach, A., Bailey, S. T., Ghosh, S. and Schmid, J. A. (2004). Cytosolic, nuclear and nucleolar localization signals determine subcellular distribution and activity of the NF-kappaB inducing kinase NIK. *J. Cell Sci.* **117**, 3615-3624.

Carlotti, F., Dower, S. K. and Qwarnstrom, E. E. (2000). Dynamic shuttling of nuclear factor kappa B between the nucleus and cytoplasm as a consequence of inhibitor dissociation. *J. Biol. Chem.* **275**, 41028-41034.

DeLotto, R. (2001). Gastrulation defective, a complement factor C2/B-like protease, interprets a ventral prepattern in Drosophila. *EMBO Rep.* **2**, 721-726.

Drier, E. A., Huang, L. H. and Steward, R. (1999). Nuclear import of the Drosophila Rel protein Dorsal is regulated by phosphorylation. *Genes Dev.* **13**, 556-568.

Drier, E. A., Govind, S. and Steward, R. (2000). Cactus-independent regulation of Dorsal nuclear import by the ventral signal. *Curr. Biol.* **10**, 23-26.

Foe, V. E. and Alberts, B. M. (1983). Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in Drosophila embryogenesis. *J. Cell Sci.* **61**, 31-70.

Foe, V. E., Field, C. M. and Odell, G. M. (2000). Microtubules and mitotic cycle phase modulate spatiotemporal distributions of F-actin and myosin II in Drosophila syncytial blastoderm embryos. *Development* **127**, 1767-1787.

Frescas, D., Mavrakis, M., Lorenz, H., Delotto, R. and Lippincott-Schwartz, J. (2006). The secretory membrane system in the Drosophila syncytial blastoderm embryo exists as functionally compartmentalized units around individual nuclei. *J. Cell Biol.* **173**, 219-230.

Fuchs, J. P., Giloh, H., Kuo, C. H., Saumweber, H. and Sedat, J. (1983). Nuclear structure: determination of the fate of the nuclear envelope in Drosophila during mitosis using monoclonal antibodies. *J. Cell Sci.* **64**, 331-349.

Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M. and Nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390**, 308-311.

Ghosh, S. and Baltimore, D. (1990). Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B. *Nature* **344**, 678-682.

Ghosh, S. and Karin, M. (2002). Missing pieces in the NF-kappaB puzzle. *Cell* **109**, S81-S96.

Gillespie, S. K. and Wasserman, S. A. (1994). Dorsal, a Drosophila Rel-like protein, is phosphorylated upon activation of the transmembrane protein Toll. *Mol. Cell. Biol.* **14**, 3559-3568.

Handwerger, K. E., Murphy, C. and Gall, J. G. (2003). Steady-state dynamics of Cajal body components in the Xenopus germinal vesicle. *J. Cell Biol.* **160**, 495-504.

Hashimoto, C., Gerttula, S. and Anderson, K. V. (1991). Plasma membrane localization of the Toll protein in the syncytial Drosophila embryo: importance of transmembrane signaling for dorsal- ventral pattern formation. *Development* **111**, 1021-1028.

Kiseleva, E., Rutherford, S., Cotter, L. M., Allen, T. D. and Goldberg, M. W. (2001). Steps of nuclear pore complex disassembly and reassembly during mitosis in early Drosophila embryos. *J. Cell Sci.* **114**, 3607-3618.

la Cour, T., Kiemer, L., Molgaard, A., Gupta, R., Skriver, K. and Brunak, S. (2004). Analysis and prediction of leucine-rich nuclear export signals. *Protein Eng. Des. Sel.* **17**, 527-536.

Leptin, M. (1991). twist and snail as positive and negative regulators during Drosophila mesoderm development. *Genes Dev.* **5**, 1568-1576.

Li, X. and Stark, G. R. (2002). NFkappaB-dependent signaling pathways. *Exp. Hematol.* **30**, 285-296.

Minakhina, S., Yang, J. and Steward, R. (2003). Tamo selectively modulates nuclear import in Drosophila. *Genes Cells* **8**, 299-310.

Morisato, D. and Anderson, K. V. (1995). Signaling pathways that establish the dorsal-ventral pattern of the Drosophila embryo. *Annu. Rev. Genet.* **29**, 371-399.

Moussian, B. and Roth, S. (2005). Dorsoventral axis formation in the Drosophila embryo – shaping and transducing a morphogen gradient. *Curr. Biol.* **15**, R887-R899.

Nicolas, F. J., De Bosscher, K., Schmierer, B. and Hill, C. S. (2004). Analysis of Smad nucleocytoplasmic shuttling in living cells. *J. Cell Sci.* **117**, 4113-4125.

Phair, R. D. and Misteli, T. (2001). Kinetic modelling approaches to in vivo imaging. *Nat. Rev. Mol. Cell Biol.* **2**, 898-907.

Pierce, J. W., Lenardo, M. and Baltimore, D. (1988). Oligonucleotide that binds nuclear factor NF-kappa B acts as a lymphoid-specific and inducible enhancer element. *Proc. Natl. Acad. Sci. USA* **85**, 1482-1486.

Reach, M., Galindo, R. L., Towb, P., Allen, J. L., Karin, M. and Wasserman, S. A. (1996). A gradient of cactus protein degradation establishes dorsoventral polarity in the Drosophila embryo. *Dev. Biol.* **180**, 353-364.

Rorth, P. (1998). Gal4 in the Drosophila female germline. *Mech. Dev.* **78**, 113-118.

Roth, S., Stein, D. and Nusslein-Volhard, C. (1989). A gradient of nuclear localization of the dorsal protein determines dorsoventral pattern in the Drosophila embryo. *Cell* **59**, 1189-1202.

Roth, S., Hiromi, Y., Godt, D. and Nusslein-Volhard, C. (1991). cactus, a maternal gene required for proper formation of the dorsoventral morphogen gradient in Drosophila embryos. *Development* **112**, 371-388.
Rothwarf, D. M. and Karin, M. (1999). The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus. *Sci. STKE* **1999**, RE1.
Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of Drosophila with transposable element vectors. *Science* **218**, 348-353.
Rusch, J. and Levine, M. (1994). Regulation of the dorsal morphogen by the Toll and torso signaling pathways: a receptor tyrosine kinase selectively masks transcriptional repression. *Genes Dev.* **8**, 1247-1257.
Rushlow, C. A., Han, K., Manley, J. L. and Levine, M. (1989). The graded distribution of the dorsal morphogen is initiated by selective nuclear transport in Drosophila. *Cell* **59**, 1165-1177.
Smith, C. L. and DeLotto, R. (1994). Ventralizing signal determined by protease activation in Drosophila embryogenesis. *Nature* **368**, 548-551.
Stathopoulos, A. and Levine, M. (2002). Dorsal gradient networks in the Drosophila embryo. *Dev. Biol.* **246**, 57-67.
Steward, R. (1989). Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. *Cell* **59**, 1179-1188.
Steward, R., Zusman, S. B., Huang, L. H. and Schedl, P. (1988). The dorsal protein is distributed in a gradient in early Drosophila embryos. *Cell* **55**, 487-495.
Towb, P., Galindo, R. L. and Wasserman, S. A. (1998). Recruitment of Tube and Pelle to signaling sites at the surface of the Drosophila embryo. *Development* **125**, 2443-2450.
Yang, J. and Steward, R. (1997). A multimeric complex and the nuclear targeting of the Drosophila Rel protein Dorsal. *Proc. Natl. Acad. Sci. USA* **94**, 14524-14529.