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# MICROINJECTION OF AN NM23 SPECIFIC ANTIBODY INHIBITS CELL DIVISION IN RAT EMBRYO FIBROBLASTS

S.M. Sorscher[1,2*], P. Steeg[3], J.R. Feramisco[1,2,4], C. Buckmaster[2], G.R. Boss[1] and J. Meinkoth[1,2]

[1]Departments of Medicine, [4]Pharmacology and [2]Cancer Center, University of California, San Diego, La Jolla, CA 92093

[3]National Cancer Institute, Laboratory of Pathology, Bethesda, MD

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**Summary:** Expression of the nm23/NDP-K gene correlates with reduced metastasis in some tumors and with increased proliferation in both nontransformed and transformed cells in culture. Decreased nm23/NDP-K expression results in mitotic arrest in neuroblasts of developing Drosophila. In order to better understand the biological role(s) of nm23 in non-transformed cells, an nm23-specific antibody was introduced into rat embryo fibroblasts and effects on DNA synthesis and cell cycle progression were analyzed. Microinjection of the nm23 antibody inhibited cell division with no apparent effect on DNA synthesis. Control experiments revealed that the survival of cells injected with the nm23 antibody was similar to that of control antibody injected cells in the absence of cell division. These results suggest that in mammalian fibroblasts, as in Drosophila, nm23 expression may be necessary for progression through the cell cycle. 

The non metastasizing or "nm" 23 gene was identified by subtractive hybridization of cell lines of differing metastatic potential derived from a single mouse melanoma (K-1735) (1). Highly metastatic lines expressed less nm23 mRNA than cell lines of lower metastatic potential, suggesting that the nm23 gene functions as a metastatic suppressor gene. Consistent with this hypothesis, transfection of an nm23 cDNA into highly metastatic melanoma cell lines (2), or into the human MDA-MB-435 breast carcinoma cell line (3), reduced the metastatic potential of these cell lines.

The predicted amino acid sequence of the first human nm23 gene cloned (nm23-H1) was shown to be identical to the A subunit of erythrocyte nucleoside diphosphate kinase (NDP-K) (4). NPD-K is a hexameric enzyme which converts nucleoside and deoxynucleoside diphosphates to their corresponding triphosphates (5), and is composed of A and B subunits which share 88% amino acid identity (4). A second human gene, nm23-H2, was isolated and found to encode a predicted amino acid sequence identical to the B subunit of the human NDP-K (4, 6). Consistent with these results, both recombinant nm23-H1 and H2 demonstrate NDP-K activity (7).

*To whom correspondence should be addressed. Fax: (619) 534-7286.

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Many potential roles for NDP-K have been suggested including regulation of cellular processes which depend upon nucleotide triphosphate pools such as DNA synthesis and G-protein mediated signal transduction (8-12). Expression of nm23/NDP-K has been associated with differentiation in mouse organogenesis (13) and proliferation in peripheral blood lymphocytes (14).

Recent data suggests that NDP-K appears active in normal tubulin functioning (15, 16). Tubulin is the major protein component of microtubules, which function in a diverse array of cellular processes including chromosome segregation at mitosis. The regeneration of GTP, required for microtubule assembly, appears to rely on NDP-K (15-27). Genetic evidence supports a role for nm23/NDP-K in microtubule function. Null mutations in the abnormal wing development (awd) gene, which encodes the microtubule-associated NDP-K in Drosophila, result in mitotic arrest in neuroblasts of these insects (16).

To better understand the biological roles of nm23, rat embryo fibroblasts (REF 52) were injected with an antibody raised to nm23 and DNA synthesis and cell cycle progression were assessed. Interestingly, injection of the nm23 antibody had no effect on serum-stimulated DNA synthesis, but far fewer fibroblasts injected with nm23 antibody were able to divide. These results are qualitatively similar to nm23 null mutations in Drosphila, where decreased NDP-K activity results in mitotic arrest, and suggests that nm23 function is required for cell division in fibroblasts.

## Materials and Methods

Cells and Cell Culture: Rat embryo fibroblasts (REF 52) were propagated in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin and streptomycin at 37° in an atmosphere of 5% $CO_2$. Cells to be injected were plated on glass coverslips, grown to 50-80% confluence and injected into the cytoplasm as previously described (28). For synchronization in $G_0$, cells were incubated in DMEM containing 0.05% FBS for 48 h prior to microinjection. For synchronization at the $G_1$/S boundary, the cells were incubated in isoleucine-deficient DMEM for 36 h followed by DMEM containing 10% FCS and 10 $\mu$g/ml aphidicholine for 24 h. DNA synthesis was assessed by incorporation of the thymidine analog, bromodeoxyuridine (BrdU) and its subsequent detection by indirect immunofluorescence (28).

Antibody Preparation: Affinity purified anti nm23-peptide 11 antibody (ab 11) was raised to the proposed internal hydrophilic region of the nm23-H1 protein corresponding to amino acids 297 to 348 and has been characterized previously (29). Antibody 11 was concentrated to approximately 800 $\mu$g/ml in .5 x PBS. Affinity purified rabbit IgG (Jackson Laboratories) was injected at 0.8-2 mg/ml in .5 x PBS as a control for the effects of injection.

Western Blot Analysis: A 100 mm plate of logarithmic REF52 cells was solubilized in 200 $\mu$l of 5X Laemmli sample buffer. After gentle pipetting the sample was centrifuged for 10 min at 10, 000 x g. The supernatant was heated at 90°C for 5 min and a 20$\mu$l aliquot was electrophoresed on a 12.5% SDS-polyacrylamide gel. The gel was transferred to nitrocellulose and incubated with ab11. A secondary biotinylated rabbit antibody (1.5 mg/ml; Vector) and streptavidin alkaline phosphatase (Biorad) were applied. Colorimetric detection was by NBT/BCIP staining (Biorad).

Measurement of NDP-K Activity: Cells were washed once in situ with phosphate buffered saline (PBS) and scraped into PBS, 1 mM EDTA. Following centrifugation at 10,000 x g for 15 sec, the cells were extracted by sonicating in 50 mM Tris HCL, pH 7.4, 1 mM $MgCl_2$, 1 mM DTT (extract buffer). The extracts were centrifuged at 10,000 x g at 4° C for 15 min and the supernatants diluted five-fold in extract buffer prior to measuring NDP-K activity. The enzyme assay contained in a final volume of 15 $\mu$l, 75 mM Tris HCL, pH 7.4, 10 mM $MgCl_2$ , 5 mM ATP, 1 mM GDP,

10 mM β-glycerol phosphate, 10 μM $Na_3VO_4$ , 0.1 μCi [γ-$^{32}$P] ATP and 0.1 μg of extracted protein. The assay was initiated by substrate addition and terminated by adding 3 μl of 12 M formic acid. A 15 μl aliquot of the reaction mixture was spotted on fluor-impregnated cellulose TLC plates pre-spotted with non-radioactive ATP and GTP and the plates were developed for 3 h in saturated ammonium sulfate/water/3 M sodium acetate pH 5.5/10 N sodium hydroxide/2-propanol (80/10/6/2/2) (v:v). The spots corresponding to ATP and GTP were cut out and radioactivity was quantified by liquid scintillation counting. In this TLC system, GTP (the reaction product) has an $R_f$ value of 0.85 and ATP (the reaction substrate) an $R_f$ value of 0.6.

Immunofluorescence: DNA synthesis was assessed using indirect immunofluorescence with a monoclonal antibody raised to BrdU (Amersham) as previously described (28). Injected cells were identified by staining with a fluorescein (FITC)-conjugated anti-rabbit secondary antibody (Cappell) diluted 1:100 in 0.5 % Nonidet P-40, PBS, 1 mg/ml BSA. Cells were counted in a blinded fashion using a Zeiss Axiophot fluorescence microscope and a 40 X (1.3NA) oil immersion lens.

## Results

**REF52 cells express the nm23 protein and contain abundant NDP kinase activity.** In order to verify that REF52 cells expressed the nm23 protein, total cell extracts were prepared from exponentially growing REF52 cells. Following electrophoresis and transfer to nitrocellulose, the membranes were incubated with either ab11 or rabbit IgG as a control. The ab 11 identified a 17kDa protein, the predicted size of the nm23/NDP-K gene product (Figure 1).

As reported in other cell systems (5, 15, 30), REF 52 cells contained abundant NDP-K activity: 4.5 +/- 0.05 μmol/min/mg protein (mean ± SD of four independent experiments preformed in duplicate). Inclusion of ab11 in these same experiments, using 1:3 to 1:1000 dilutions of the concentrated antibody (800 μg/ml), had no inhibitory effect on NDP-K activity in vitro. However, as microinjected antibodies may inhibit intracellular protein function by means other than simple inhibition of enzymatic activity (i.e. steric hinderance, sequestration, etc.), the biological effects of microinjection of the nm23-specific antibody were examined.

**Effects of nm23 antibody injection on DNA synthesis.** To investigate any requirement for nm23 function in DNA replication, the effects of nm23 antibody injection on DNA synthesis in REF 52 cells were examined. In initial experiments, exponentially growing cells were injected with ab11 or affinity purified rabbit IgG (as a control). Following injection, the cells were incubated in growth medium containing BrdU for 24 h, after which the cells were fixed and DNA synthesis assessed by indirect immunofluorescence (28). Representative fields of cells are shown in Figure 2. Injection of ab11 resulted in a slight reduction (15%) in the number of cells synthesizing DNA compared to uninjected cells on the same coverslip. In these experiments, 900 ab11 injected cells on 12 coverslips (6 independent experiments) were analyzed, and the effect of ab11 injection was seen in 12 of 12 injected coverslips.

To better assess the effect of ab11 on DNA synthesis, ab11 was injected into synchronized REF 52 cells. Two methods of synchronization were used: serum deprivation to arrest cells in $G_0$ and isoleucine deprivation followed by apidicholine treatment to arrest cells at the $G_1$/S boundary. The kinetics of DNA synthesis in synchronized cells are shown in Table 1. Following serum deprivation, DNA synthesis occurs between 16-28 h after serum addition. In cells synchronized by isoleucine deprivation/aphidicholine treatment, DNA synthesis occurs between 8 -16 h after removal of aphidicholine.

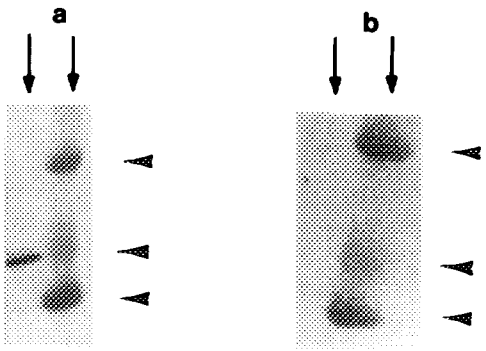


**Figure 1.** REF52 cells express nm23.
Western blot analysis of total cell protein incubated with ab11 (panel a, left lane) and control rabbit IgG (panel b, left lane). Prestained molecular weight markers (right lanes) at 43, 29 and 18 kD are indicated. Ab11 reacted only with a 17kDa protein corresponding to the molecular weight of nm23/NDP-K.

Table 2 demonstrates that injection of ab11 had no significant effect on DNA synthesis in synchronized cells. When cells were synchronized, injected in $G_0$, and labeled during the first S phase following serum stimulation, there was no apparent reduction in the number of ab11 injected cells which synthesized DNA (relative inhibition 4.6%) compared to uninjected cells on the same coverslip. In these experiments, 630 antibody injected cells on 8 coverslips were analyzed. Similarly, when cells were arrested and injected at the $G_1$/S boundary and labeled during the first S phase following removal of aphidicholine, there was no significant reduction in the number of cells which synthesized DNA (relative "inhibition" -7%). In these experiments, 101 cells on 2 coverslips were analyzed.

While there was a lack of an effect of ab11 injection on DNA synthesis during the first S phase, if the injected cells were allowed to proceed through a second S phase, ab 11-injected cells exhibited a 19% decrease in the percentage of labeled nuclei (218 cells analyzed), similar to the decrease observed in exponentially growing cells. Together, these results suggested that the decreases in DNA synthesis observed might be related to an effect on progression through the cell cycle.

**Effect of nm23 antibody injection on cell division.** To determine if cell cycle progression was affected by injection of ab11, REF 52 cells were synchronized at the $G_1$/S boundary, injected and then incubated in growth medium for 24 h, a time sufficient for the cells to have divided. The cells were then fixed and stained for the presence of injected antibody, and the number of cells present at the end of the experiment was scored. Since approximately 80% of injected REF52 cells survive microinjection (see below), the maximum expected recovery of cells would be 160% if every cell divided. This is in good agreement with the recovery of contol-injected cells (159%) (Table 3). In contrast, the recovery of ab11 injected cells was much lower. These results demonstrate that nearly all of the control-injected cells divided, while far fewer of the ab11-injected cells divided.

To establish that the inhibitory effect of ab11 on cell division was unrelated to the synchronization process , exponentially growing cells were injected and the number of cells

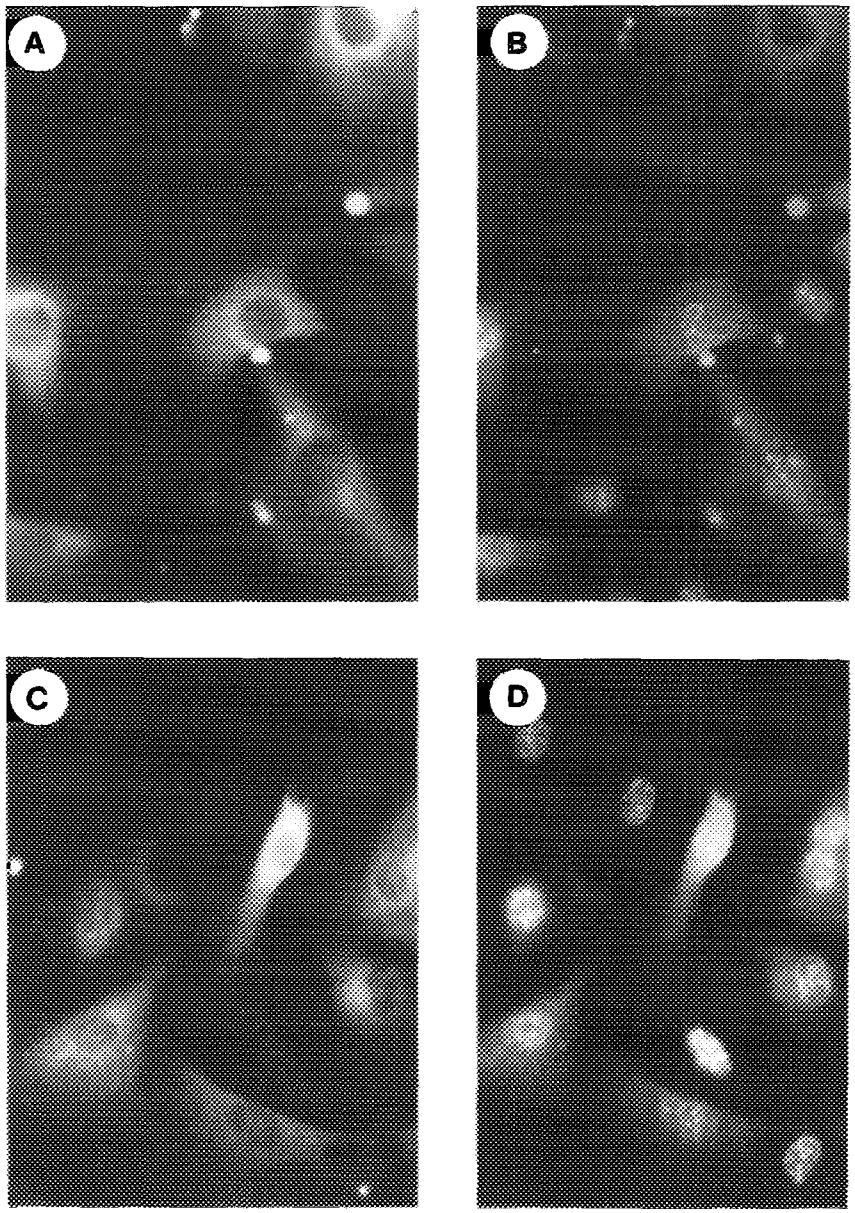


**Figure 2.** DNA synthesis in Ab11 and control injected REF52.
Representative fields of ab11 injected cells (A, B) and control injected cells (C, D) are shown. Panels A and C are fluorescence photomicrographs which illustrate injected cells (white cytoplasm). Panels B and D are fluorescence photomicrographs illustrating cells which synthesize DNA (labeled nuclei).

recovered after 30 to 48 h was assessed. Using these conditions, 112% of ab11-injected cells and 150% of IgG-injected cells were recovered (Table 3). To establish that the reduced recovery was not due to a non-specific toxic effect of ab11, serum-starved REF52 cells were injected with ab11 or control antibody and stimulated with growth medium for as long as possible (26 h) without an intervening division. Under these conditions, the survival of ab11-injected (69%, n-895) and

Table 1 KINETICS OF DNA SYNTHESIS IN SYNCHRONIZED REF52 CELLS

| Serum Starvation | | | Isoleucine/Aphidicolin | | |
|---|---|---|---|---|---|
| Labeling period[a] (h) | % labeled nuclei | (N)[b] | Labeling period (h) | % labeled nuclei | (N) |
| 0 - 4 | 0 | 100 | 0 - 4 | 0 | 100 |
| 4 - 8 | 0 | 100 | 4 - 8 | 3 | 103 |
| 8 - 16[c] | 9 | 100 | 8 - 12 | 40 | 100 |
| 16 - 20 | 73 | 100 | 12 - 16 | 71 | 105 |
| 20 - 24 | 50 | 100 | 16 - 20 | 4 | 100 |
| 24 - 28 | 21 | 101 | 20 -30[d] | 44 | 102 |
| 28 - 32 | 1 | 112 | | | |

[a] - Isoleucine/Apidicolin synchronization and serum starvation techniques are described in the Methods sections.
[b] - N=number of cells examined.
[c] - an 8 hour labelling period was used.
[d] - a 10 hour labelling period was used.

control-injected cells (72%, n-576) was essentially the same. Thus, the difference in cell recovery observed in the ab11-injected cells does not appear due to a toxic effect of ab11.

**Discussion.** In many tumor types, nm23 expression has been shown to be reduced in more advanced or metastatic, compared to primary tumors, including breast (31-35), hepatocellular (36), and gastric carcinomas (37), though there was no prognostic significance to nm23 expression in lung adenocarcinoma (38). Also, while in neuroblastoma (39) and in colon cancer (40) there was no detectable change in nm23 expression with more advanced disease, in both of these tumors alterations in the nm23 gene have been demonstrated (41-43)

Altered levels of nm23 expression imply that this gene product somehow affects the metastatic potential of malignant cells. However, the mechanism(s) through which nm23 influences metastatic behavior remains unclear. With the demonstration of the identity of the predicted amino acid sequence of nm23 to NDP-K (4), attention has focused on identifying the biological role(s) of nm23/NDP-K in normal cells in order to better understand how altered nm23 expression might affect malignant cells. Changes in nm23 expression have been demonstrated during proliferation and differentiation in both normal and transformed cells. For example, during mouse

Table 2 ANTIBODY 11 INJECTION DOES NOT REDUCE SERUM-STIMULATED DNA SYNTHESIS IN REF52 CELLS

| Synchronization[a] Techniques | Antibody [b] Injected | Injected Cells Analyzed (N) | BrdU in Injected (%) | BrdU in Uninjected (%) | Relative [c] Inhibition (%) |
|---|---|---|---|---|---|
| Serum Starvation | nm23-ab11 | 65 | 94 | 95 | 1 |
| | | 82 | 96 | 97 | 1 |
| | | 91 | 85 | 88 | 3.4 |
| Isoleucine Starvation/ Aphidicolin Treatment | nm23-ab11 | 50 | 92 | 84 | -9.5 |
| | | 51 | 92 | 88 | -4.5 |

[a] - Synchronization techniques are described in the Methods section.
[b] - The nm23 antibody (ab11) was injected at 0.8 $\mu$g/ml.
[c] - Relative inhibition = [% uninjected cells (BrdU +) - % ab 11 injected cells (BrdU +)] / % uninjected cells ( BrdU +).

Table 3 EFFECT OF NM23 ANTIBODY OR CONTROL ANTIBODY INJECTION ON CELL CYCLE PROGRESSION

| Synchronization [a] | Antibody Injected | Injected Cells Analysed (N) | Recovery (%) + s.d.[c] |
|---|---|---|---|
| Isoleucine/Aphidicolin | nm23-ab11 | 1076 | 107 ± 2.4 |
| | Rabbit IgG | 689 | 159 ± 2.2 |
| None [b] | nm23-ab11 | 273 | 112 ± 1.1 |
| | Rabbit IgG | 212 | 150 ± 1.1 |

[a] - Cells were synchronized as described in the methods section.
[b] - Exponentially growing REF 52 cells.
[c] - As approximately 80% of injected cells survive injection, if all cells divided the maximum recovery would be between 160%.

embryogenesis, nm23 protein accumulation appears to occur at the same time as differentiation in multiple different epithelial tissues (13). In mitotically-stimluated peripheral blood lymphocytes, nm23 protein levels increase in parallel with DNA synthesis, and correlate with increases in the expression of proliferation markers (14, 44, 45). Consistent with these results, inhibition of lymphocyte proliferation with cyclosporin results in reduced proliferation and NDP-K protein expression (14). In addition, NDP-K has been shown to copurify with ribonucleotide reductase, an enzyme critical for DNA synthesis (12), suggesting that NDP-K might be important in the regulation of DNA synthesis in normal cells.

Microinjection of an nm23-specific antibody had no effect on the ability of injected REF 52 cells to synthesize DNA. In cells synchronized using two independent techniques, there was no effect of ab11 on the number of cells replicating DNA. However, ab11 significantly reduced the number of cells able to proceed through mitosis. This effect was observed both in cells previously synchronized and released from the $G_1$/S boundary and in exponentially growing cells. In both cases, the recovery of control-injected cells (150-158%) was much higher than cells injected with ab11 (107-112%). A non-specific toxic effect of ab11 injection seems unlikely since: 1) there was no inhibitory effect of ab11 on the ability of injected cells to synthesize DNA; and 2) there was no effect of ab11 on cell recovery when cells were incubated for as long as 26 h without an intervening division. Under these conditions, the survival of ab 11-injected and control-injected antibody cells was essentially the same. Rather, the experimental results suggest that ab11 interferes with the ability of injected cells to divide.

In light of these results, the small reduction in DNA synthesis (15%) in exponentially growing cells, compared to uninjected controls, is likely due to the inability of some of the nm23 antibody injected cells to complete cell division, and proceed to S phase. This is supported by experiments in synchronized cells where there was no effect of ab11 injection on DNA synthesis during the first S phase, but a 19% reduction in DNA synthesis when the cells were required to divide before DNA synthesis was assessed.

The mechanism by which ab11 inhibits cell cycle progression remains unclear, since in vitro NDP-K activity was not reduced by ab11. The lack of an effect on kinase activity in vitro is not due to a lack of antibody specificity. Ab11 has been shown in the past to recognize nm23/NDP-K

protein by immunostaining (16, 35), as well as by immunoblots using cell extracts from K-1735 melanoma cell lines (2) . Furthermore, in REF 52, ab11 recognized a 17kD protein, which corresponds to the molecular weight of nm23/NDP-K.

It remains possible that ab11 reduces NDP-K activity in vivo, perhaps exerting its effect on a specific cellular pool of NDP kinases. Alternatively, ab11 may affect nm23 protein localization or turnover. There is accumulating evidence that kinase activity is not responsible for some of the biological effects of nm23/NDP-K (16). For example, Drosophila carrying the killer mutation of the prune gene and specific awd gene mutations (Awd $^{K\text{-}pr}$) demonstrate profound developmental abnormalities (46) , although the awd gene product retains full NDP-K catalytic activity (47). Also, Golden recently found no significant difference in NDP-K activity in K-1735 melanoma cells of differing metastatic potential which were expressing transfected nm23/NDP-K (48). Finally, while several studies have demonstrated reduced nm23 expression in metastatic relative to primary breast tumors, reduced NDP-K activity does not correlate with lymph node involvement in breast cancer (32-35, 49).

The apparent ability of the anti-nm23 antibody to inhibit cell division in REF 52 cells is similar to the consequences of NDP-K deficiency in Drosophila. The nm23 gene product is highly homologous to the product of the Drosophila awd gene, an NDP-K shown to associate with microtubules (16, 29). Cells derived from larvae with null mutantions in the awd gene lack 98% of the NDP-K activity and are arrested in metaphase (16). Similar to antibodies raised to the awd gene product, ab11 recognizes a 17 kDa protein isolated from purified microtubules. Thus it seems likely that both the awd and nm23 gene products function, at least in part, as microtubule-associated NDP-K (16, 50). While Biggs and others have shown that microtubules copurify with nm23/NDP-K and the copurified protein contains kinase activity (16), others have reported no direct phosphorylation of tubulin-bound GDP in vitro, and that NDP-K does not bind microtubules in the presence or absence of microtubule associated proteins (51). Also, no significant difference in tubulin organization was seen in K-1735 TK melanoma cells transfected with nm23 expression vectors (48). In preliminary experiments, we have seen no difference in tubulin staining between nm23 and control-antibody injected cells (data not shown). However, these experiments were preformed in exponentially growing cells, and effects on tubulin immunostaining may only become apparent during specific stages of the cell cycle. In addition to tubulin, NDP-K has been shown to associate physically with a number of other proteins (15, 52) including the elongation factor EF-$\alpha$ (53), the heterotrimeric G-proteins, $G_0$ and $G_s$, (54, 55) and the ras related G-proteins (52, 53). Aberration of any of these associations could potentially explain the effect of ab11 on cell cycle progression. Finally, these studies were performed using nontransformed fibroblasts in culture and their relevance to malignant and metastatic cells in vivo is not known. Additional studies will help clarify the role of the nm23 gene product in cell division.

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