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Cell Cycle Regulation of c-Jun N-Terminal Kinase Activity at the Centrosomes

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The c-Jun N-terminal kinase (JNK), a subgroup of the mitogen-activated protein kinase (MAPK) family of serine/threonine kinases, has established functions in cell growth and apoptosis. While the mechanisms are unclear, JNK has also been also implicated in signaling pathways that initiate cell cycle checkpoints and cell cycle progression. By following the localization of active and inactive JNK during the cell cycle, we have found that the majority of cellular JNK is soluble and present in the cytoplasm and the nucleus. Interestingly, insoluble fractions of JNK are also localized in nuclear and cytoplasmic speckles, and to the centrosomes. While JNK is associated with the centrosome throughout the cell cycle, it is only active at the centrosome from S phase through anaphase. This novel localization of centrosomal JNK is a possible link between JNK-activating stimuli and centrosome or cell cycle events.

Key Words: **JNK; kinase; centrosome; cell cycle; localization; pericentriolar matrix; immunofluorescence.**

The c-Jun N-terminal kinases (JNKs) are a subgroup of the mitogen-activated protein kinase (MAPK) family of serine/threonine kinases, which also includes the extracellular signal-regulated kinase (ERK) and the stress-activated kinase p38. JNK is activated by mitogenic stimuli (e.g., Ras, epidermal growth factor), cellular stress (e.g., hyperosmolarity, heat shock, oxidative stress), and DNA-damaging agents (e.g., ultraviolet radiation, γ radiation, genotoxic drugs) (1). There are 10 isoforms of human JNK generated by alternative splicing of 3 genes, *Jnk1*, *Jnk2*, and *Jnk3* (2). JNK1 and JNK2 isoforms are ubiquitously expressed, while JNK3 expression is restricted to the central nervous system (1). JNK1 contributes to antigen-induced apoptosis of T cells in adult mice, and apoptotic responses to γ radiation and UV radiation (3, 4). However, JNK2 appears to play a role in the regulation of T-cell proliferation and differentiation in adult mice (5). JNK1 and JNK2 have recently been shown to have essential and redundant functions in development (6). Single JNK1 or JNK2 null mice are viable, while the double JNK1/JNK2 null phenotype is embryonically lethal (4–6).

While JNK has an established role in stress and DNA damage-induced apoptosis, recent evidence suggests it may also be involved in cell cycle control. JNK contributes to, but is not sufficient for, the transcription of cyclin D1 in response to Src kinase (7). Cyclin D1 complexes with Cdk4 or Cdk6 to initiate the G1/S phase transition (8). This suggests that JNK participates in cell cycle progression stimulated by growth factor signaling pathways that activate Src kinase. In addition, JNK interacts with and is inhibited by the cyclin dependent kinase (cdk) inhibitor, $p21^{Waf1/Cip1}$ (9, 10). This interaction is disrupted at the initiation of DNA synthesis and correlates with an increase in JNK activity during S phase (10). JNK is also involved in the initiation of the S phase checkpoint in response to proteasome inhibition by LLnL (11).

These results suggest that JNK functions during S phase to regulate cell cycle progression. Recently, the two other members of the MAPK family, stress-activated kinase p38 and extracellular signal-regulated kinase (ERK), have been implicated in mitotic control. The p38 kinase is essential for the spindle assembly checkpoint in *Xenopus* egg extracts and is activated by disruption of the spindle in mammalian cells (12). ERK is also essential for the spindle assembly checkpoint in *Xenopus* egg extracts but may play a different role than p38 in mammalian cells (13, 14). ERK localizes to kinetochores and spindle microtubules during somatic cell mitosis (15, 16). It also interacts with and phosphorylates the kinesin-like motor

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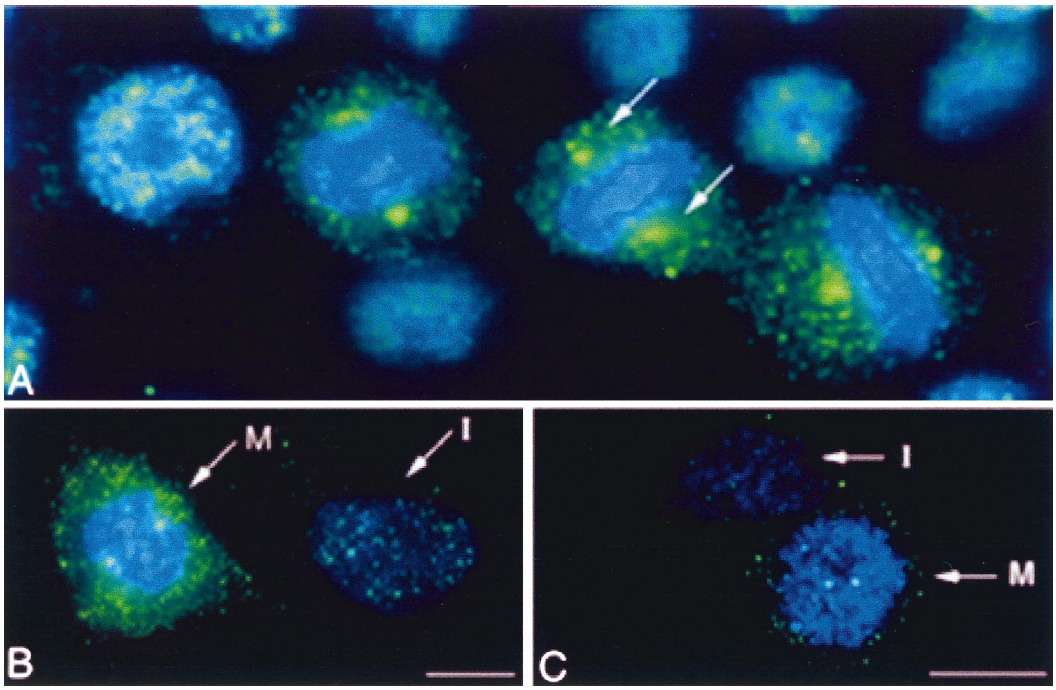

FIG. 1. Phospho-JNK is present in detergent-insoluble cellular structures. HeLa cells were grown on polylysine-coated coverslips and fixed in 4% ultrapure formaldehyde (A). To detect the localization of active JNK, cells were incubated with anti-phospho JNK antibody #9251 (green) and DAPI DNA stain (blue), and then visualized by fluorescence microscopy (A) or deconvolution fluorescence microscopy (B and C). To extract soluble protein, cells were incubated in 0.5% Triton X-100 for 2 min prior to fixation (C). Arrows point to the centrosomal pattern of staining. Representative interphase (I) and mitotic (M) cells images are shown.

protein CENP-E, which appears to function in prometaphase and anaphase chromosome movement (16).

The regulation of mitotic events by MAPK family members p38 and ERK suggests that JNK may also have mitotic functions. To determine if JNK was involved in mitotic regulation, we examined the localization of active and inactive JNK during the cell cycle. We found that JNK was localized to centrosomes using immunofluorescence and subcellular fractionation. While JNK was localized to centrosomes throughout the cell cycle, it was only active in this compartment beginning near the initiation of centrosome duplication at the G1/S phase transition. JNK remained active at the centrosomes until late anaphase. The timing of active JNK localization suggests that JNK may participate in centrosome duplication and/or the regulation of centrosome-associated events.

MATERIALS AND METHODS

Antibodies. The primary antibodies used for immunofluorescence were: anti-phospho JNK rabbit antibody (#9251, 1:100 dilution) specific for the active form of JNK was purchased from New England Biolabs (Beverly, MA), anti-phospho JNK monoclonal antibody (anti-p-JNK(G-7), 1:100) specific for the active form of JNK was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-JNK rabbit antibody (anti-JNK1(FL), 1:100) raised to full-length recombinant JNK1 was purchased from Santa Cruz Biotechnology, Inc., anti-JNK2 rabbit antibody (anti-JNK2(N-18), 1:100) raised to amino-terminal peptide of JNK2 was purchased from Santa Cruz Biotechnology Inc., anti-γ tubulin monoclonal antibody (1:100) was purchased from Sigma-Aldrich Corp. (St. Louis, MO), anti-β tubulin monoclonal antibody (1:20) (17), and human autoimmune serum (#4171, 1:2000) specific for proteins of the pericentriolar matrix (18). The secondary antibodies used for immunofluorescence were: goat anti-human, rabbit, and mouse IgG (H + L) conjugated to Alexa488 purchased from Molecular Probes (Eugene, OR), conjugated to FITC, Texas Red, and Cy5 purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). The primary antibodies used for Western blotting were: anti-γ tubulin mouse monoclonal antibody purchased from Sigma–Aldrich Corp. (St. Louis, MO), anti-JNK rabbit antibody (Ab101) (19), anti-JNK rabbit antibody (Ab7193) prepared as described (19) and peptide purified using the Sulfolink Kit from Pierce (Rockford, IL), anti-p38 rabbit antibody (C-20), anti-ERK rabbit antibody (C-16) and anti-Bcl-$X_{S/L}$ rabbit antibody (L-19) purchased from Santa Cruz Biotechnology Inc., anti-aldolase goat antibody purchased from Biodesign International (Saco, ME), anti-GRP78 rabbit antibody purchased from StressGen Biotechnologies (Collegeville, PA), anti-lamin B1 mouse monoclonal antibody purchased from Zymed Laboratories (San Francisco, CA), and anti-Golgin97 mouse monoclonal antibody purchased from Molecular Probes.

Cell culture. HeLa human cervical carcinoma cells were maintained in DMEM containing 10% fetal bovine serum (FBS), 10 mM Hepes (pH 7.4), penicillin (100 units/ml), and streptomycin (100 μg/ml). For all experiments, cells were plated on acid-etched, polylysine-coated glass coverslips. Plated cells were maintained at 37°C in 5% CO_2.

Immunofluorescence. As recently described in detail (20), coverslips were washed in 0.5% Triton X-100 for 2 min on ice and fixed in 4% ultrapure formaldehyde (Polysciences, Inc.) in PEM buffer (80

mM K-Pipes [pH 7.6], 5 mM EGTA, 2 mM $MgCl_2$) for 10–20 min on ice. For immunofluorescence of β-tubulin, 4% polyethylene glycol was added to PEM buffer during the permeabilization and fixation steps. Coverslips were then washed with PEM buffer and permeabilized in 0.5% Triton X-100 in PEM buffer for 30 min. Coverslips were washed with PEM buffer and blocked in 2.5% nonfat dry milk in TBST (50 mM Tris [pH 7.6], 150 mM NaCl, 0.1% Tween 20) overnight. Coverslips were then incubated for 1 h at 37°C with primary antibodies diluted in TBST, washed in TBST, and incubated for 1 h at 37°C with secondary antibodies diluted 1:200 in TBST. After washing in TBST, coverslips were counterstained with 0.4 μg/ml of 4,6 diamino-2-phenylindole (DAPI) (Molecular Probes) in TBST and mounted with VectashieldR antifade medium (Vector Laboratories, Burlingame, CA) or ProLong antifade medium (Molecular Probes). Figures are composite images obtained with a Deltavision, deconvolution-based optical workstation (Applied Precision, Issaquah, WA). Z-series stacks of multiple focal planes were used to render 3-D volumes.

Centrosome purification. Centrosomes were isolated from HeLa cells essentially as described (21) with the following modifications. HeLa cells (6×10^7) were treated with 0.2 μM nocodazole (Sigma) and 1 μg/ml cytochalasin D (Sigma) for 1 h at 37°C. All subsequent steps were at 4°C. Cells were washed sequentially with 1× TBS (50 mM Tris [pH 7.6], 150 mM NaCl), and 0.1× TBS/8% sucrose. Cells were resuspended in 2 ml of 0.1× TBS/8% sucrose, and then 8 ml of fractionation lysis buffer was added (1 mM Hepes [pH 7.2], 0.5% NP-40, 0.5 mM $MgCl_2$, 0.1% β-mercaptoethanol, 3.3 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 0.5 mM NaF). This suspension was passed 10 times through a serological pipette to aid cell lysis. Lysate was centrifuged twice at 2500*g* for 10 min. The supernatant was filtered through a 70 μm nylon filter and incubated on ice after addition of Hepes and DNase 1 to final concentrations of 10 mM and 1 μg/ml, respectively. The lysate was underlain with 60% sucrose in sucrose dilution buffer (10 mM Piperazine-*N*,*N*′-bis(ethanesulfonic acid) (Pipes) [pH 7.2], 0.1% Triton X-100, 0.1% β-mercaptoethanol) and centrifuged for 1.5 h at 10,000*g*. The bottom 2 ml of the spun lysate was retained, vortexed, loaded onto a discontinuous 40%/50%/70% sucrose gradient, and centrifuged for 1.5 h at 120,000*g*. Fractions were collected, diluted to 1 ml with PEM buffer, and centrifuged at 15,000 rpm in a microfuge for 30 min to pellet centrosomes. Centrosome pellets were washed twice with PEM buffer and resuspended in Laemmli sample buffer (Bio-Rad, Hercules, CA) containing 5% β-mercaptoethanol.

Western blotting. Samples were heated at 100°C for 10 min and proteins were separated by SDS–PAGE. Proteins were transferred to nitrocellulose membranes, and membranes were blocked in 10% nonfat dry milk in TBST. Membranes were incubated for 1 h in 5% nonfat dry milk in TBST containing primary antibody, washed 3 times for 7 min in TBST, and then incubated in 5% nonfat dry milk in TBST containing HRP-conjugated secondary antibody. Signal was developed using ECL reagents (Amersham Life Science, Inc., Piscataway, NJ).

BrdU labeling. Replicating DNA was labeled by incubating cultures in medium containing the thymidine analog 5-bromodeoxyuridine (BrdU, Sigma) at 20 μM. Incorporated BrdU was detected by immunofluorescence using mouse monoclonal anti-BrdU antibody with nuclease (Amersham).

RESULTS

Localization of Active JNK to Detergent-Insoluble Structures

We investigated the localization of JNK during the cell cycle to gain insight into a possible function in the cell cycle. To determine the localization pattern of active JNK relative to nuclear structures, we chose an antibody that detects the active form of JNK (New England Biolabs). This antibody was raised to a specific phosphopeptide spanning the JNK activation motif, Thr(183)-Pro(184)-Tyr(185) in human JNK1. Rabbit serum raised to this phosphopeptide was purified by two rounds of negative selection with the nonphosphorylated peptide followed by elution from a phosphorylated peptide affinity column. Using this antibody, we observed punctate nuclear staining and a centrosomal pattern of staining (Fig. 1). We further resolved this staining by extracting soluble protein with detergent prior to fixation and using deconvolution microscopy. The nuclear speckles and cytoplasmic foci remained after detergent extraction, showing a stable association of active JNK with components of the cytoskeleton and nuclear matrix (Fig. 1).

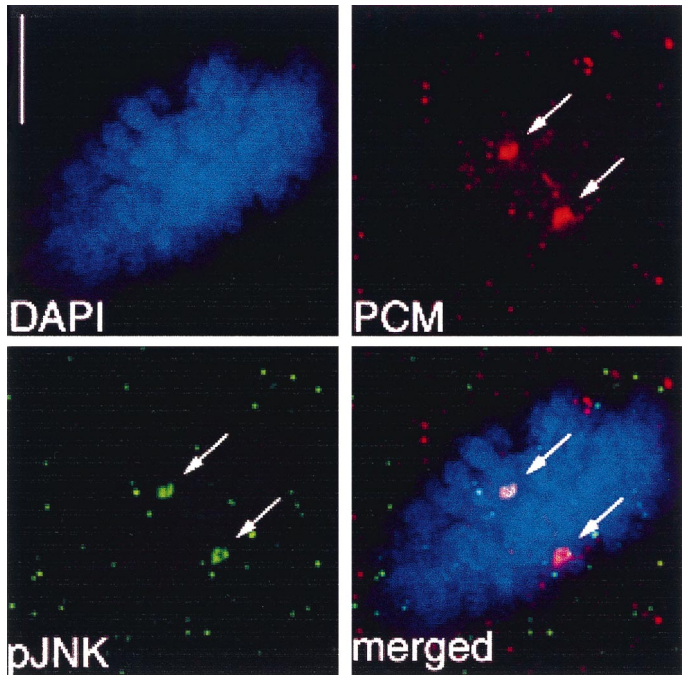

FIG. 2. Phospho-JNK is a component of the centrosome. HeLa cells were grown on polylysine-coated coverslips, extracted in 0.5% Triton X-100 for 2 min, and fixed in 4% ultrapure formaldehyde. To detect centrosomal localization of active JNK, fixed cells were incubated with human autoimmune serum #4171 (PCM; red), anti-phospho JNK antibody G-7 (pJNK; green), and DAPI DNA stain (DAPI; blue). Panels PCM, pJNK, and DAPI were merged, to identify areas of colocalization (merged; pink). One representative metaphase cell is shown.

Active JNK Localizes to the Pericentriolar Matrix of Centrosomes

The focal pattern of staining with anti-phospho JNK antibody in detergent-extracted cells suggested that JNK may be localized to centrosomes during inter-

phase and/or mitosis. To test this hypothesis, HeLa cells were co-stained with anti-phospho JNK antibody and human autoimmune serum specific for antigens in the pericentriolar matrix (PCM) (Fig. 2). Merging of the individual images shows that JNK is a component of the centrosome (Fig. 2, merged panel; colocalization at centrosomes appears pink).

To determine if JNK was present in the pericentriolar matrix and/or centriole core of the centrosome, HeLa cells were costained with anti-phospho JNK antibody, mouse anti-γ tubulin (present in the centrioles), and human autoimmune serum specific for pericentriolar antigens (Fig. 3). Merging of the individual images shows that JNK is a component of the pericentriolar matrix. The γ-tubulin staining partially overlaps with JNK staining but occupies a discrete region of the centrosome.

We also used a biochemical approach to confirm that JNK is localized to centrosomes. First, we purified centrosomes from HeLa cells by discontinuous sucrose gradient ultracentrifugation, and then we Western blotted for the presence of JNK in fractions containing purified centrosome proteins. We used a well-established method for purifying centrosomes from HeLa cells (21). Briefly, HeLa cells were lysed and supernatants fractionated by two rounds of sucrose density gradient ultracentrifugation. Using this protocol, centrosomes are expected to sediment between 50 and 60% sucrose (fractions 5–7) after the final purification step. Western blots of protein in our final purification step showed a peak of γ-tubulin (a structural centrosomal protein) in fractions 4–7 as expected, confirming that we successfully isolated centrosomes with this protocol. We also checked to be sure our centrosome preparation was not contaminated with other subcellular compartments, by Western blotting the fractions of the final purification step with antibodies to marker proteins from other subcellular compartments. No markers from other subcellular compartments were detected in the purified centrosome fractions 4–7 including GRP78 (endoplasmic reticulum marker), lamin B1 (nuclear marker), aldolase (soluble cytoplasmic marker), Bcl-X_L (mitochondrial marker) and Golgin 97 (Golgi apparatus marker) (Fig. 4). However, a peak of JNK protein was detected in fractions 4–7, confirming the presence of JNK at the centrosome. We also checked for the presence of the other MAPK family members, ERK and p38, in these fractions. In most cases, ERK and p38 were undetectable (Fig. 4). However, ERK was detectable in some fractionations in very low levels relative to the levels in the whole cell lysate (data not shown). This is consistent with reports of ERK localization to the centrosome (19, 20), but suggests that ERK may be present at the centrosome in much lower levels or is more weakly associated with the centrosome than JNK.

Cell Cycle-Dependent Regulation of Centrosomal JNK Activity

To determine if the activity or localization of centrosomal JNK was regulated during the cell cycle, asynchronous exponentially growing HeLa cells were costained with anti-phospho JNK antibody, and human autoimmune serum to the PCM. Cell cycle stages were identified by the patterns of DNA staining, centrosome number, and centrosome position. JNK was active at the centrosomes beginning after centrosome duplication, which is known to occur near the G1 to S phase transition, and remained active through late anaphase (Fig. 5). By labeling cells actively synthesizing DNA with BrdU, we found that 98% of cells in S phase were positive for anti-phospho JNK antibody staining (data not shown). This suggests that JNK is activated before or during the G1/S phase transition.

While centrosomal JNK activity appeared to be upregulated during late G1 and downregulated during late anaphase, it remained possible that the localization rather than the activity of centrosomal JNK was cell cycle dependent. We therefore costained asynchronous HeLa cells with anti-JNK2 antibody (N-19) that detects both active and inactive JNK, PCM autoimmune serum, and DAPI (Fig. 6). We found that JNK was localized to the centrosome during all stages of the cell cycle (Figs. 5 and 6). Taken together, these data show that while JNK localization to the centrosome is cell cycle independent, JNK activity at the centrosome is cell cycle dependent.

Specificity of JNK Localization

Control experiments were performed to confirm that centrosomal staining required both primary and secondary antibodies (data not shown). We also performed additional localization experiments with a total of five JNK specific antibodies to establish specificity of the centrosomal localization. The JNK specific antibodies used include: peptide-affinity purified anti-phospho JNK rabbit antibody (New England Biolabs), anti-JNK rabbit antibody #9251 raised to full length recombinant JNK1 (Santa Cruz), rabbit antibody raised to the N-terminus of JNK2 (anti-JNK2(N-18), Santa Cruz), a monoclonal antibody specific for the active form of JNK (anti-p-JNK(G-7), Santa Cruz), and peptide affinity-purified anti-JNK rabbit antibody (Ab7193) produced in our laboratory. In all cases, these antibodies detected JNK at the centrosomes (Figs. 3 and 5, and data not shown) thereby confirming our conclusion that JNK is present at centrosomes.

DISCUSSION

Here we have shown for the first time, that JNK localizes to centrosomes in addition to cytoplasmic and

nuclear speckle compartments. This finding furthers our recent understanding that JNK is regulated by localization, in addition to expression and phosphorylation regulation mechanisms. In addition to our finding that JNK is localized to centrosomes and nuclear speckles, JNK has been reported to localize to both the endoplasmic reticulum and mitochondria with location specific functions separate from the well-characterized stores of cytoplasmic JNK (22–24). The nuclear speckles detected with our antibodies most likely represent JNK localization to interchromatin granule clusters and/or sites of active transcription, consistent with its known functions in transcriptional regulation. We also found that JNK activity at the centrosome is cell cycle dependent, suggesting that the centrosomal JNK may play a role in regulating cell cycle and/or centrosome events. Interestingly, we found that centrosomal JNK was specifically localized to the pericentriolar matrix (PCM), the site of nucleation of spindle microtubules (25). Several possible functions are consistent with this finding based on the known functions of other centrosomal kinase and centrosomal proteins that have been functionally linked to JNK.

Fyn, a member of the Src family of tyrosine kinase and an upstream activator of JNK, has also been shown to localize to centrosomes and to the cleavage furrow during cytokinesis (26, 27). Fyn functions to promote S phase entry in response to platelet-derived growth factor (PDGF) (28). Although the mechanism for S phase control remains unclear, Fyn has recently been shown to interact with a subunit of the dynein motor complex that regulates centrosome separation during mitosis (27, 29). Our evidence that centrosomal JNK is active from the G1/S transition through anaphase would be consistent with centrosomal JNK functions in the centrosomal Fyn pathways that promote S phase entry and centrosome separation during mitosis.

In addition, p53, a transcription factor and JNK substrate, localizes to the centrosome from late prophase through telophase (30, 31). The effect of p53 phosphorylation by JNK is unclear, however JNK activation is correlated with disruption of p53/JNK interactions and stabilization of p53 protein (31, 32). p53 is known to upregulate the expression of the cell cycle inhibitor, $p21^{Waf1/Cip1}$ in response to DNA damage. The inhibition of Cdk2/cyclin complexes by $p21^{Waf1/Cip1}$ arrests both the cell cycle in G1 and the centrosome cycle before duplication at the G1/S phase transition (33). Thus, the activation of centrosomal JNK may release p53 sequestered at the centrosome and contribute the inhibition of centrosome duplication by DNA damage.

Members of the HSP70 family may also link centrosomal JNK to extracellular stimuli. HSP70 is the major inducible heat shock protein and is known to inhibit JNK activity (34–36). Hsp70 has been shown to temporally localize to the centrosome during cell division but not interphase when expression is induced (36). HSP70 plays a central role in the recovery of centrosome and spindle structure after mild heat shock damage (37). This raises the possibility that inhibition of centrosomal JNK by HSP70 contributes to the repair of heat shock-induced damage to centrosomes. In this model, centrosomal JNK activity positively regulates centrosome function, and its inhibition by HSP70 would result in the arrest of centrosome function, to allow for repair. Alternatively, JNK activity may inhibit a centrosome 'checkpoint'-inducing substrate such that inhibition of JNK results in the activation of a centrosome checkpoint to allow for repair.

A role for JNK in the response to extracellular stresses such as DNA damage and heat shock, is most consistent with the known functions of JNK. While JNK may contribute to cell cycle regulation in adult tissues, studies of JNK null mice suggest that JNK does not play an essential role in normal cell division during development. JNK1 and JNK2 are ubiquitously expressed in mouse embryonic tissue. However, while the JNK1/JNK2 null phenotype is embryonically lethal, the lethality is due to abnormalities in brain development, while other tissues develop normally through embryonic day 11 (6). On the other hand, JNK is activated by multiple stimuli, including epidermal growth factor (EGF), nocodazole, ultraviolet radiation, γ radiation, osmotic shock, anisomycin, oxidative stress, and heat shock (1). These stimuli regulate the cell cycle directly or through stress or DNA-damage repair pathways. Recently cell cycle control has been linked to centrosome cycle control, enhancing our understanding of the coordination of these events. As discussed previously, DNA damage has also recently been shown to activate a centrosomal checkpoint as well as cell cycle checkpoints (33). Given that JNK is activated by multiple environmental stimuli that affect the centrosome cycle and stress responses, it is likely that centrosomal JNK acts as a sensor of or sensitizer to environmental stimuli.

The coordination of the cell cycle with centrosomal events is essential for the maintenance of genomic integrity. In cases where these processes are uncoupled, endoreduplication and aneuploidy can result from abnormal partitioning of chromosomes to daughter cells. The development of aneuploidy has long been recognized as a major contributing factor to tumorigenesis (39, 40). The regulation of centrosome events has recently resurfaced as proteins such as Cdk2, with a clearly established function in cell cycle regulation, are being found to have additional functions in centrosome processes. This study suggests that this may also be true for JNK, a kinase with a well established role in stress and DNA-damage responses. The majority of cellular JNK is soluble and likely to be responsible for signaling pathways that coordinate cellular stress

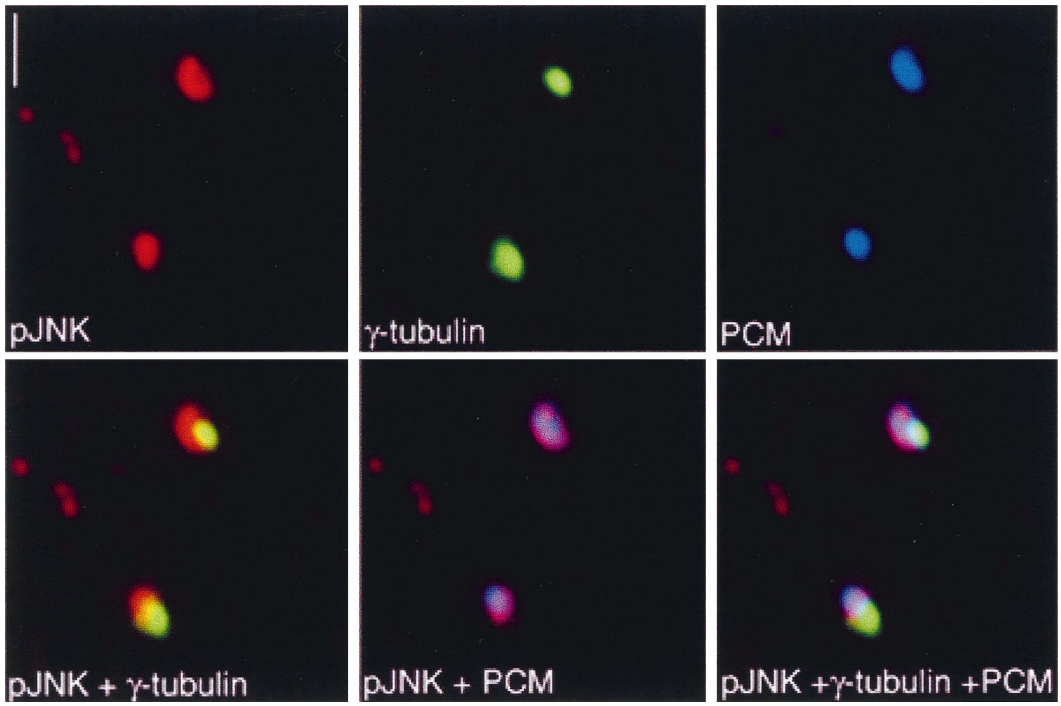

FIG. 3. JNK is a component of the pericentriolar matrix. HeLa cells were grown on polylysine-coated coverslips, extracted in 0.5% Triton X-100 for 2 min and fixed in 4% ultrapure formaldehyde. To detect subcentrosomal localization of active JNK, fixed cells were incubated with anti-phospho JNK antibody #9251 (pJNK; red), monoclonal anti-γ-tubulin antibody (γ-tubulin; green), and human autoimmune serum #4171 (PCM; blue). One representative metaphase cell is shown. Panels were merged as indicated in the lower three panels. Colocalization of anti-phospho JNK with human autoimmune serum appears purple.

stimuli with stress responses. However, we have described an insoluble fraction of JNK that is associated with centrosomes and regulated during the cell cycle. Future studies will determine if this fraction of JNK can also be regulated by cellular stress signals and what role JNK plays in centrosome events.

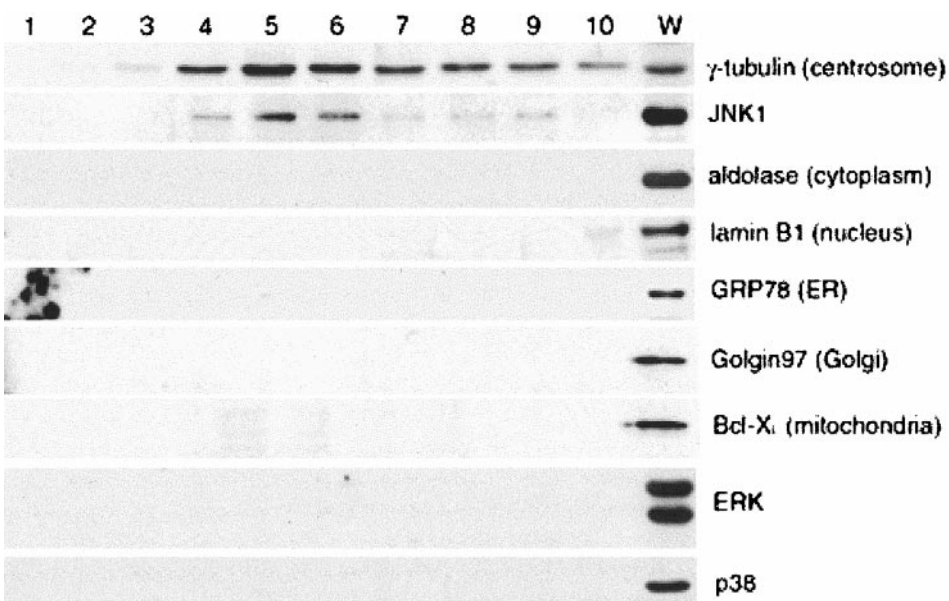

FIG. 4. JNK is copurified with centrosomes. Centrosomes were prepared from 6×10^7 HeLa cells and purified on a discontinuous sucrose gradient. 10% of protein recovered from each fraction and 5 μg of HeLa whole cell lysate (W) were Western blotted for the presence of JNK, ERK, p38 and subcellular compartment markers: γ-tubulin (centrosome), aldolase (cytosol), lamin B1 (nucleus), GRP78 (ER, endoplasmic reticulum), Golgin 97 (Golgi), and Bcl-X_L (mitochondria).

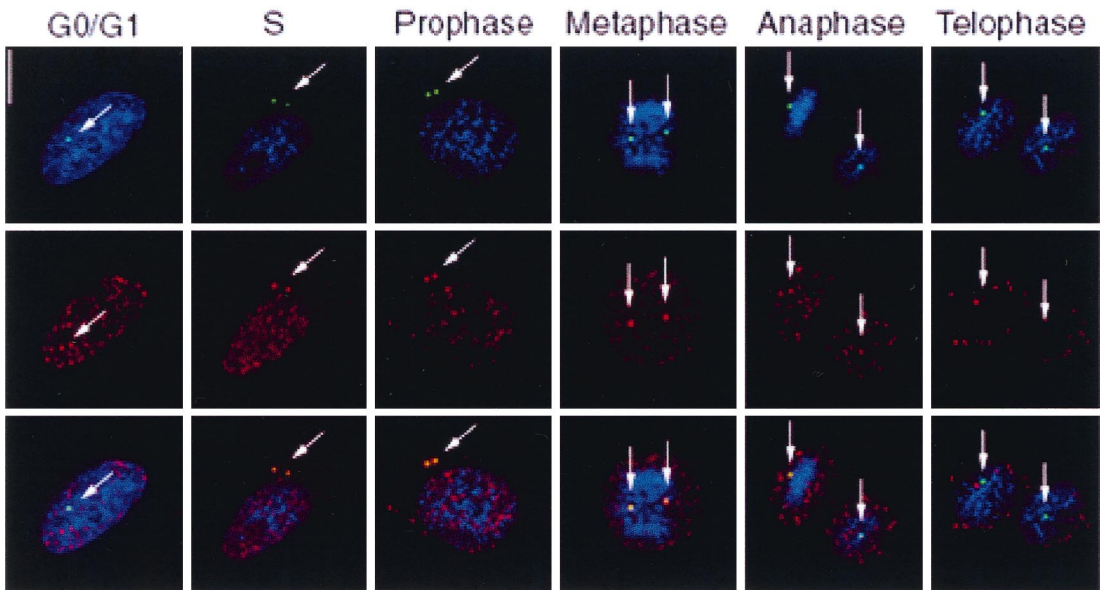

FIG. 5. Centrosomal JNK activity is cell-cycle regulated. HeLa cells were grown on polylysine-coated coverslips, incubated in 0.5% Triton X-100 for 2 min, and fixed in 4% ultrapure formaldehyde. Cells were then incubated with anti-phospho JNK antibody #9251 (pJNK; red), human autoimmune serum #4171 (PCM; green), and DAPI DNA stain (DAPI; blue). Images were overlaid to gauge colocalization (merged; yellow). Representative cells from G0/G1, S, prophase, metaphase, anaphase and telophase are shown. Arrows indicate the position of centrosomes.

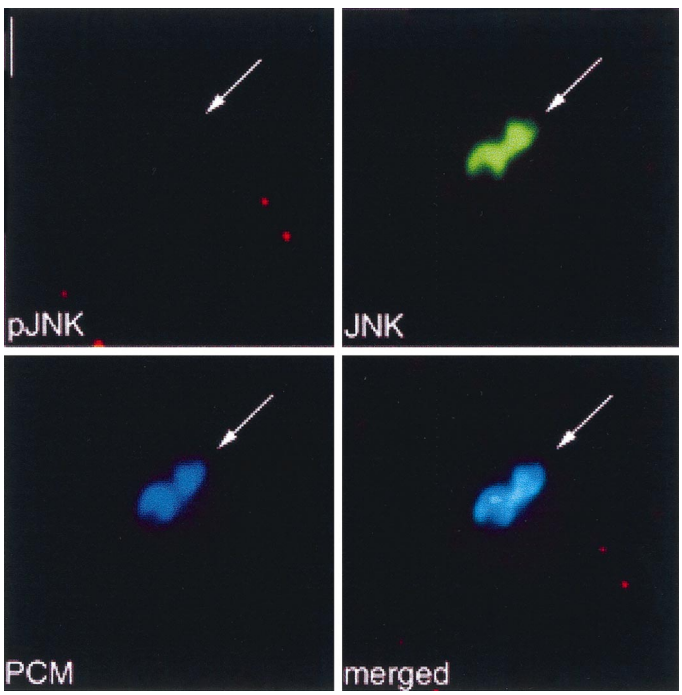

FIG. 6. JNK is present but not active at the centrosome during G0/G1. HeLa cells were grown on polylysine-coated coverslips, incubated in 0.5% Triton X-100 for 2 min, and fixed in 4% ultrapure formaldehyde. Fixed cells were incubated with anti-phospho JNK antibody #9251 (pJNK; red), human autoimmune serum #4171 (PCM, blue) and anti-JNK2 antibody N-18. One representative interphase cell is shown. Arrows indicate the position of the centrosome. Panels (pJNK, JNK and PCM) were merged to show colocalization of JNK with PCM staining (merged; light blue).

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REFERENCES

1. Chen, Y.-R., and Tan, T.-H. (2000) The c-Jun N-terminal kinase pathway and apoptotic signaling. *Int. J. Oncol.* **16,** 651–662.
2. Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanaugh, J., Sluss, H. K., Derijard, B., and Davis, R. J. (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. *EMBO J.* **15,** 2760–2770.
3. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) JNK1: A protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76,** 1025–1037.
4. Dong, C., Yang, D. D., Wysk, M., Whitmarsh, A. J., Davis, R. J., and Flavell, R. A. (1998) Defective T cell differentiation in the absence of Jnk1. *Science* **282,** 2092–2095.
5. Yang, D. D., Conze, D., Whitmarsh, A. J., Barrett, T., David, R. J., Rincon, M., and Flavell, R. A. (1998) Differentiation of CD4+ T cells to Th1 cells requires MAP kinase JNK2. *Immunity* **9,** 575–585.
6. Kuan, C. Y., Yang, D. D., Samantha, R. D. R., Davis, R. J., Rakic, P., and Flavell, R. A. (1999) The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron* **22,** 667–676.
7. Lee, R. J., Albanese, C., Stenger, R. J., Watanabe, G., Inghirami,

G., Haines, K., III, Webster, M., Muller, W. J., Brugge, J. S., Davis, R. J., and Pestel, R. G. (1999) pp60(v-src) induction of cyclin D1 requires collaborative interactions between the extracellular signal-regulated kinase, p38, and Jun kinase pathways. A role for cAMP response element-binding protein and activating transcription factor-2 in pp60(v-src) signaling in breast cancer cells. *J. Biol. Chem.* **274,** 7341–7350.

8. Barnes, D. M., and Gillett, C. E. (1998) Cyclin D1 in breast cancer. *Breast Cancer Res. Treat.* **52,** 1–15.
9. Shim, J., Lee, H., Park, J., Kim, H., and Choi, E-J (1996) A non-enzymatic p21 protein inhibitor of stress-activated protein kinases. *Nature* **381,** 804–806.
10. Patel, R., Bartosch, B., and Blank, J. L. (1998) p21WAF1 is dynamically associated with JNK in human T-lymphocytes during cell cycle progression. *J. Cell Sci.* **111,** 2247–2255.
11. Tchou, W. W., Yie, T. A., Tan, T.-H., Rom, W. N., and Tchou-Wong, K. M. (1999) Role of c-Jun N-terminal kinase 1 (JNK1) in cell cycle checkpoint activated by the protease inhibitor *N*-acetyl-leucinyl-leucinyl-norleucinal. *Oncogene* **18,** 6974–6980.
12. Takenaka, K., Moriguchi, T., and Nishida, E. (1998) Activation of the protein kinase p38 in the spindle assembly checkpoint and mitotic arrest. *Science* **280,** 599–602.
13. Minshull, J., Sun, H., Tonks, N. K., and Murray, A. W. (1994) A MAP kinase-dependent spindle assembly checkpoint in Xenopus egg extracts. *Cell* **79,** 475–486.
14. Takenaka, K., Gotoh, Y., and Nishida, E. (1997) MAP kinase is required for the spindle assembly checkpoint but is dispensable for the normal M phase entry and exit in *Xenopus* egg cell cycle extracts. *J. Cell Biol.* **136,** 1091–1097.
15. Shapiro, P. S., Vaisberg, E., Hunt, A. J., Tolwinski, N. S., Whalen, A. M., McIntosh, J. R., and Ahn, N. G. (1998) Activation of the MKK/ERK pathway during somatic cell mitosis: Direct interactions of active ERK with kinetochores and regulation of the mitotic 3F3/2 phosphoantigen. *J. Cell Biol.* **142,** 1533–1545.
16. Zecevic, M., Catling, A. D., Eblen, S. T., Renzi, L., Hittle, J. C., Yen, T. J., Gorbsky, G. J., and Weber, M. J. (1998) Active MAP kinase in mitosis: Localization at kinetochores and association with the motor protein CENP-E. *J. Cell Biol.* **142,** 1547–1558.
17. Fuller, G. M., Brinkley, B. R., and Boughter, J. M. (1975) Immunofluorescence of mitotic spindles by using monospecific antibody against bovine brain tubulin. *Science* **187,** 948–950.
18. Mack, G. J., Rees, J., Sandblom, O., Balczon, R., Fritzler, M. J., and Rattner, J. B. (1998) Autoantibodies to a group of centrosomal proteins in human autoimmune sera reactive with the centrosome. *Arthritis Rheum.* **41,** 551–558.
19. Chen, Y.-R., Meyer, C. F., and Tan, T.-H. (1996) Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in γ radiation-induced apoptosis. *J. Biol. Chem.* **271,** 631–634.
20. Van Hooser, A., and Brinkley, B. R. (1999) Methods for *in situ* localization of proteins and DNA in the centromere-kinetochore complex. *In* Methods in Cell Biology (D. M. Prescott., Ed.), Vol. 61, Academic Press, New York.
21. Wigley, W. C., Fabunmi, R. P., Lee, M. G., Marino, C. R., Muallem, S., DeMartino, G. N., and Thomas, P. J. (1999) Dynamic association of proteasomal machinery with the centrosome. *J. Cell Biol.* **145,** 481–490.
22. Pena, E., Berciano, M. T., Fernandez, R., Crespo, P., and Lafarga, M. (2000) Stress-induced activation of c-Jun N-terminal kinase in sensory ganglion neurons: Accumulation in nuclear domains enriched in splicing factors and distribution in perichromatin fibrils. *Exp. Cell Res.* **256,** 179–191.
23. Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., and Ron, D. (2000) Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* **287,** 664–666.
24. Kharbanda, S., Saxena, S., Yoshida, K., Pandey, P., Kaneki, M., Wang, Q., Cheng, K., Chen, Y.-N., Campbell, A., Sudha, T., Yuan, Z.-M., Narula, J., Weichselbaum, R., Nalin, C., and Kufe, D. (2000) Translocation of SAPK/JNK to mitochondria and interaction with Bcl-x_L in response to DNA damage. *J. Biol. Chem.* **275,** 322–327.
25. Lacey, K. R., Jackson, P. K., and Stearns, T. (1999) Cyclin-dependent kinase control of centrosome duplication. *Proc. Natl. Acad. Sci. USA* **96,** 2817–2822.
26. Ley, S. C., Marsh, M., Bebbington, C. R., Proudfoot, K., and Jordan, P. (1994) Distinct intracellular localization of Lck and Fyn protein tyrosine kinases in human T lymphocytes. *J. Cell Biol.* **125,** 639–649.
27. Campbell, K. S., Cooper, S., Dessing, M., Yates, S., and Buder, A. (1998) Interaction of $p59^{fyn}$ kinase with the dynein light chain, Tctex-1, and colocalization during cytokinesis. *J. Immunol.* **161,** 1728–1737.
28. Roche, S., Fumagalli, S., and Courtneidge, S. A. (1995) Requirement for Src family protein tyrosine kinases in G2 for fibroblast cell division. *Science* **269,** 1567–1569.
29. Barton, N. R., and Goldstein, L. S. B. (1996) Going mobile: Microtubule motors and chromosome segregation. *Proc. Natl. Acad. Sci. USA* **93,** 1735–1742.
30. Morris, V. B., Brammall, J., Noble, J., Reddel, R. (2000) p53 localizes to the centrosomes and spindles of mitotic cells in the embryonic chick epiblast, human cell lines, and a human primary culture: An immunofluorescence study. *Exp. Cell Res.* **256,** 122–130.
31. Fuchs, S. Y., Adler, V., Pincus, M. R., and Ronai, Z. (1998) MEKK1/JNK signaling stabilizes and activates p53. *Proc. Natl. Acad. Sci. USA* **95,** 10541–10546.
32. Fuchs, S. Y., Adler, V., Buschmann, T., Yin, Z., Wu, X., Jones, S. N., and Ronai, Z. (1998) JNK targets p53 ubiquitination and degradation in nonstressed cells. *Genes Dev.* **12,** 2658–2663.
33. Tarapore, P., Horn, H. F., Tokuyama, Y., and Fukasawa, K. (2001) Direct regulation of the centrosome duplication cycle by the p53-$p21^{Waf1/Cip1}$ pathway. *Oncogene* **20,** 3173–3184.
34. Liang, P., and MacRae, T. H. (1997) Molecular chaperones and the cytoskeleton. *J. Cell Sci.* **110,** 1431–1440
35. Gabai, V. L., Meriin, A. B., Mosser, D. D., Caron, A. W., Rits, S., Shifrin, V. I., and Sherman, M. Y. (1997) Hsp70 prevents activation of stress kinases. A novel pathway of cellular thermotolerance. *J. Biol. Chem.* **272,** 18033–18037.
36. Park, H.-S., Lee, J.-S., Huh, S.-H., Seo, J.-H., and Choi, E.-J. (2001) Hsp72 functions as a natural inhibitory protein of c-Jun N-terminal kinase. *EMBO J.* **20,** 446–456.
37. Brown, C. R., Hong-Brown, L. Q., Doxsey, S., and Welch, W. J. (1996) Molecular Chaperones and the centrosome. A role for HSP73 in centrosomal repair following heat shock treatment. *J. Biol. Chem.* **271,** 833–840.
38. Rattner, J. B. (1991) Hsp70 is localized to the centrosome of dividing HeLa cells. *Exp. Cell Res.* **195,** 110–113.
39. Brinkley, B. R., and Goepfert, T. M. (1998) Supernumerary centrosomes and cancer: Boveri's hypothesis resurrected. *Cell Motil. Cytoskel.* **41,** 281–288.
40. Duesberg, P. (1999) Are centrosomes or aneuploidy the key to cancer? *Science* **284,** 2091–2092.