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Mechanism of proliferation arrest of embryonic cells of *Xenopus* by diterpene compounds

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Abstract—Three diterpene compounds isolated from the anti-cancer herbal medicine kansui, namely, kansuinin B, 20-OD-ingenol *Z*, and 20-OD-ingenol *E*, specifically inhibited the proliferation of isolated embryonic cells from *Xenopus* embryos. We conducted a cytologic study to determine the mechanism underlying the arrest of the cellular proliferation by these compounds. While kansuinin B and 20-OD-ingenol *Z* treatment decreased the cell numbers in the S phase and the M phase substages of the cell cycle, 20-OD-ingenol *E* inhibited mitosis.

1. Introduction

The dried roots of *Euphorbia kansui* Liou are known as kansui, and they have been used as anti-viral and anti-cancer agents.[1,2] Focusing our attention on the diterpene compounds in kansui, we isolated various terpene compounds from the dried roots of the plant that inhibited the cell growth of embryonic cells and cancer cells.[3–6] Three of the diterpene compounds had different inhibitory effects on tumor proliferation, and one diterpene specifically inhibited the proliferation of mammary tumor cells.[6] In recent years, the cell cycle and its regulation have been extremely well studied, and it has been established that a universally operating system in eukaryotes constitutes the key player in the control of passage of the cells from one phase of the cell cycle to the next. During the cell cycle, the duplication of the genome occurring in the S phase, and the segregation of the two sets of replicated chromosomes during the M phase are critical steps. A number of observations suggest that cyclin A–Cdk2 complexes are required for the initiation of S phase.[7,8] Entry into the M phase is controlled by the M phase-promoting factor, MPF, which consists of the Cdc2 kinase and cyclin B, and the signaling transduced by the kinase allows the progression of the cells into the prophase.[9] During the subsequent metaphase–anaphase transition, the cyclin is abruptly destroyed by the ubiquitin-dependent proteolytic pathway, which results in the inactivation of MPF and the exit of the cells from the mitotic cycle.[10,11] To understand how the three diterpene compounds (Fig. 1) inhibit the cell cycle, we studied the effects of these compounds on the cell in the S phase and M phase using *Xenopus* embryos whose cells show only alternating S and M phases.

2. Results and discussion

2.1. Inhibition of cellular proliferation

The in vitro inhibitory activity of three compounds isolated from the roots of *Euphorbia kansui*, namely,

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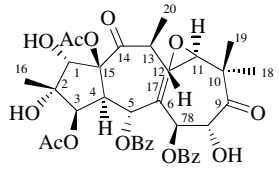
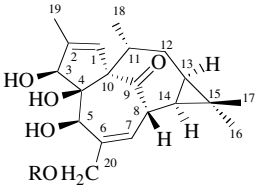


1:Kansuinin B



E/Z

2: 20-OD-ingenol Z, R=CO-(CH=CH)$_2$-(CH$_2$)$_4$-CH$_3$

E/E

3: 20-OD-ingenol E, R=CO-(CH=CH)$_2$-(CH$_2$)$_4$-CH$_3$

Figure 1. Structures of the diterpene compounds.

20-OD-ingenol *Z* and 20-OD-ingenol *E*, which are newly isolated compounds, and kansuinin B, which is already known, against the cellular proliferation of isolated *Xenopus* embryo cells was examined (Table 1). At a concentration of 10 μg/mL, kansuinin B arrested the growth of 40% of the embryonic cells, while at the concentration of 50 μg/mL, about 85% of the embryonic cells showed growth arrest. 20-OD-Ingenol *Z* and 20-OD-ingenol *E* had a similar effect, but both were more effective at inhibiting the growth of the embryonic cells than kansuinin B. At the dose of 0.5 μg/mL, 20-OD-ingenol *Z* and 20-OD-ingenol *E* caused growth arrest of 75–80% of the embryonic cells. At the concentration of 2 μg/mL, both 20-OD-ingenol *Z* and 20-OD-ingenol *E* inhibited the proliferation of almost all of the cells. Embryonic cell lysis was not observed at any concentration of these compounds. When the isolated cells were incubated in the absence of the terpenes, about 80% of the cells showed proliferative activity.

2.2. Cytological analysis

To analyze the mechanism underlying the inhibitory effect of the terpenes against cellular proliferation, we first measured the incorporation of BrdU into the *Xenopus* embryonic cell nuclei. Figure 2 shows the incorporation of BrdU into nuclei synthesizing DNA (Fig. 2-2) and the absence of BrdU incorporation in the nuclei not synthesizing DNA (Fig. 2-1). Following treatment with kansuinin B, 23% in the total cell number showed BrdU incorporation in the nuclei, suggesting DNA replication in these cells (Table 2). On the other hand, 33% and 48% of the total cell number showed BrdU incorporation after 20-OD-ingenol *Z* and 20-OD-ingenol *E* treatment, respectively. In the control, 47% of the total cell number showed BrdU incorporation. Thus, the percentages of the cells showing BrdU incorporation after kansuinin B and 20-OD-ingenol *Z* treatment, in particular, the former, were lower than that in the control, whereas BrdU incorporation after 20-OD-ingenol *E* treatment was similar to that in the control. Then, we observed effects of the diterpene compounds on the behavior of the nuclei and the chromosomes in the *Xenopus* embryonic cells. The mitotic process in these cells is divided into the prophase (Fig. 3-1), prometaphase (Fig. 3-2), and metaphase (Fig. 3-3), and the effects of the diterpenes on these stages of mitosis were analyzed. About 31% of the total number of cells in the control showed growth arrest in the prophase, while about 17% of the total number of kansuinin B treated cells and 33–35% of the total number of the 20-OD-ingenol *Z* and 20-OD-ingenol *E* treated cells showed growth arrest in the prophase (Table 3). Thus, the percentage of cells showing growth arrest in the prophase was 54% of that in the control following kansuinin B treatment and almost the same as that in the control following 20-OD-ingenol *Z* and 20-OD-ingenol *E* treatment. Both kansuinin B and 20-OD-ingenol *Z* caused cell cycle arrest and decreased the number of cells in S phase. Furthermore, kansuinin B treatment decreased the cell numbers in the prophase and metaphase, while 20-OD-ingenol *Z* treatment decreased the cell numbers in the prometaphase and metaphase. That is to say, kansuinin B inhibited entry into S phase and prophase, and 20-OD-ingenol *Z* inhibited entry into S phase and the progress of mitosis. Cyclin A is required for S phase initiation and passage though G2;[12,13] thus, cell arrest following kansuinin B treatment may cause inhibition of the reaction cascade involving cyclin A. However, since the early embryonic cell cycle in the *Xenopus* consists of only the S and M phases[14,15] and information on how the cell cycle progresses with alternating S and M phases in early embryonic cells is

Table 1. Effects of kansuinin B, 20-OD-ingenol *Z*, and 20-OD-ingenol *E* treatment on the proliferation of cells isolated from *Xenopus* blastula

Diterpene effect	200 μg/mL		50 μg/mL		10 μg/mL		2 μg/mL		0.5 μg/mL		0.1 μg/mL	
	Arre.	Gro.	Arre.	Gro.	Arre.	Gro.	Arre.	Gro.	Arre.	Gro.	Arre.	Gro.
Kansuinin B	240	6	245	45	113	173	99	291				
Percentage of arrested cell	98%		84%		40%		25%					
20-OD-ingenol *Z*	193	0	206	12	164	7	59	5	104	23	40	74
Percentage of arrested cell	100%		94%		96%		92%		81%		35%	
20-OD-ingenol *E*	73	0	240	14	151	11	64	4	102	32	29	85
Percentage of arrested cell	100%		94%		93%		94%		76%		25%	

Isolated cells from *Xenopus* blastulae were cultured in a Terasaki plate containing a salt solution, to which kansuinin B, 20-OD-ingenol *Z*, or 20-OD-ingenol *E* had been added. After 16 h, the blastomeres were examined for cleavage under a microscope. Abbreviation, Arre: arrested cell, Gro: growing cell.

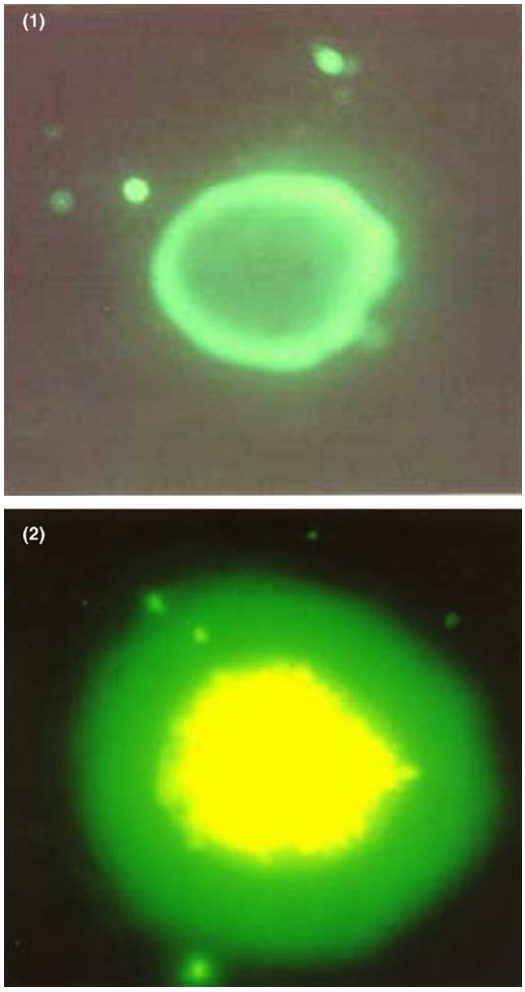


Figure 2. Incorporation of BrdU into the nucleus. Incorporation of BrdU in isolated embryonic cells of *Xenopus* was carried out for 30 min. The cells were coated with anti-BrdU-antibody and treated with Ig-fluorescein. 2-1: nucleus not showing BrdU incorporation; 2-2: nucleus showing BrdU incorporation.

still lacking, the mechanism by which kansuinin B and 20-OD-ingenol *Z* inhibit cell proliferation remain uncertain.

Following colchicine treatment, 41%, 41%, and 5.9% of the total cell number showed growth arrest in the prophase, prometaphase, and metaphase, respectively. The percentage of cells showing growth arrest in the prometaphase was about 23% of the total cell number in the control, while about 41% and 43% of the colchicine treated cells and the 20-OD-ingenol *E* treated cells showed growth arrest in prometaphase, respectively (Table 3). Thus, the number of cells showing growth arrest in the prometaphase was about 1.8 times greater in the colchicine- and 20-OD-ingenol *E*-treated group than in the control group. About 0.7% of the total number of cells in the control group showed growth arrest in the metaphase, while about 5.9% of the total number of colchicine treated cells and 3.6% of the total number of 20-OD-ingenol *E* treated cells showed growth arrest in the metaphase. On the other hand, no significant growth arrest in the metaphase was noted following kansuinin B

Table 2. Effect of kansuinin B, 20-OD-ingenol *Z*, and 20-OD-ingenol *E* treatment on incorporation of BrdU into the nucleus

Treatment		No. of S phase (%)	Total number
Control			
Exp. no.	1	64 (47)	132
	2	132 (46)	282
	3	178 (47)	377
Average of % (±SD)		47 ± 0.58	
Kansuinin B			
Exp. no.	1	73 (21)	343
	2	36 (21)	171
	3	157 (27)	564
Average of % (±SD)		23 ± 3.5	
20-OD-Ingenol Z			
Exp. no.	1	72 (28)	265
	2	108 (38)	282
	3	175 (33)	518
Average of % (±SD)		33 ± 5.0	
20-OD-Ingenol E			
Exp. no.	1	83 (49)	169
	2	252 (48)	521
	3	120 (46)	256
Average of % (±SD)		48 ± 1.5	

The isolated cells were treated with 50 μg/mL of kansuinin B, and 2 μg/mL of 20-OD-ingenol *Z* or 20-OD-ingenol *E* for 1 h. The cells were treated as described in Figure 2.

treatment or 20-OD-ingenol *Z* treatment (Table 3). Thus, growth arrest of 5.1 and 8.4 times as many cells in the metaphase was observed in the 20-OD-ingenol *E* and colchicine treated groups, respectively, as compared with that in the controls. Inhibition of cellular proliferation by 20-OD-ingenol *E* increased the number of cells that arrested in the mitosis as compared with that in the controls (Table 3). Mitotic cyclins A and B are synthesized for inducing cell entry into mitosis, and p34 cdk2–cyclin (MPF) induces the various ultrastructural changes required for cell division: namely, the breakdown of the nuclear envelope, chromatin condensation, and the construction of the mitotic spindle.[16,17] Prophase nuclei (Fig. 3-1) are distinguished from interphase nuclei by the condensation of DNA to form mitotic chromosomes, and the prometaphase nuclei are distinguished by the breakdown of the nuclear envelope (Fig. 3-2). Metaphase cells are distinguished from the cells in the other stages by the appearance of the condensed chromosomes (Fig. 3-3). The proliferation arrest by 20-OD-ingenol *E* was clearly accompanied by an accumulation of cells in the prometaphase and metaphase. Proper packaging of DNA into the chromosomes is an essential process in the preparation of cells for mitosis. Chromosome condensation at mitosis requires DNA topoisomerase II[18] and a family of proteins of highly conserved ATPase called 'structural maintenance of chromosomes' (SMCs).[19,20] Fundamental to mitotic chromosomal architecture is condensation, which in vertebrates is associated with a reduction of the chromosome length by ~100-fold relative to that in the interphase, and is crucial for physical resolution of entanglements and separation of the duplicated genome into two discrete sets.[21] Although the dynamic changes in the higher order structural changes during mitosis

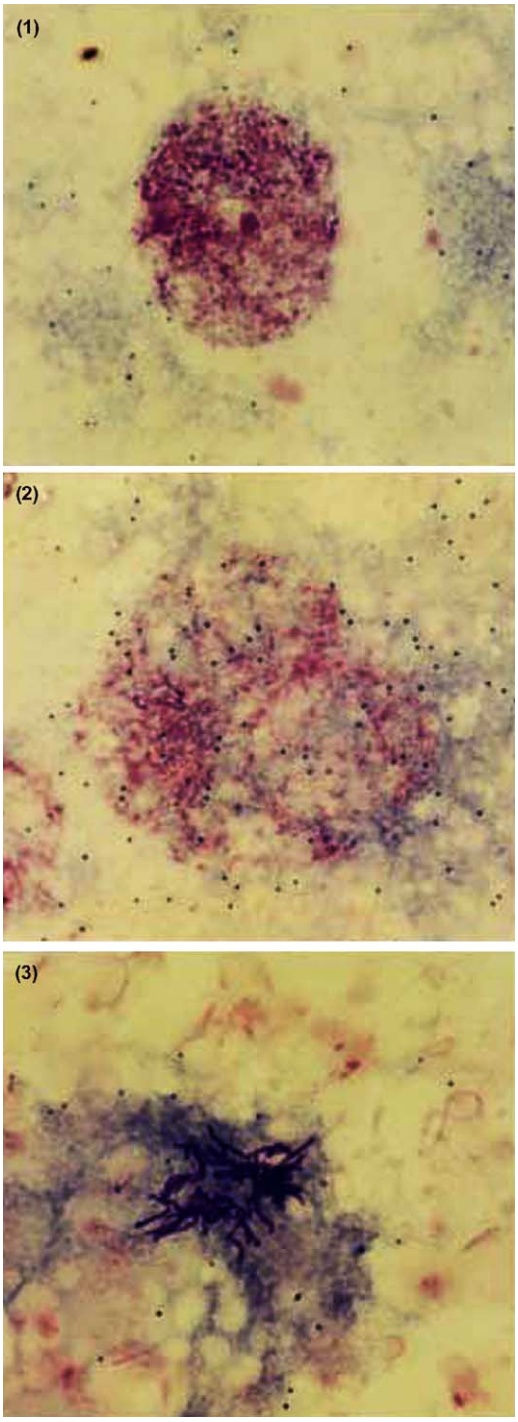


Figure 3. Nuclei or chromosomes of cells arrested in the prophase, prometaphase, and metaphase. Embryonic cells were treated with a hypotonic solution, placed on a glass slide, fixed with a solution of acetic acid–methanol and stained with Giemsa. 3-1: prophase; 3-2: prometaphase; 3-3: metaphase.

are poorly understood, arrest of proliferation by 20-OD-ingenol *E* treatment may be related to inhibition of chromatin condensation.

In conclusion, we studied how three diterpene compounds arrest cell proliferation. Both kansuinin B and 20-OD-ingenol *Z* inhibited the entrance of cells into S phase and the substages of M phase. On the other hand, 20-OD-ingenol *E* clearly inhibited the progress of mitosis.

3. Experimental

3.1. Embryos

Xenopus laevis eggs were obtained by natural amplexus of the males and females after injected of human chorionic gonadtropin (200 IU) and de-jellied with 2.5% cysteine. The embryos were then allowed to develop to stage 8 at room temperature.

3.2. Cell culture

The diterpene compounds kansuinin B (Fig. 1-1), 20-O-(2′*E*,4′*Z*-decadienoyl)ingenol (20-OD-ingenol *Z*) (Fig. 1-2), and 20-O-(2′*E*,4′*E*-decadienoyl)ingenol (20-OD-ingenol *E*) (Fig. 1-3) were dissolved with dimethylsulfoxide (DMSO). After appropriate dilution, 1 μL of each of these drugs was added to 200 μL of 2 mg/mL γ-globulin in a simple salt solution (NAM/2: 55 mM NaCl, 1 mM KCl, 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.05 mM EDTA, 1 mM $NaHCO_3$, and 1 mM sodium phosphate buffer (pH 7.5)). As a control solution, 1 μL of the same dilution of DMSO without the drug was added to 200 μL of medium. Animal cap pieces were dissected from stage 8 blastulae. Single cells from the inner surface of the pieces were separated by directing a gentle stream of a calcium- and magnesium-free medium (50 mM phosphate buffer containing 35 mM NaCl and 1 mM KCl, pH 7.0). Two or three cells were transplanted into a well of a Terasaki microculture plate containing 10 μL of medium and cultured in the presence or absence of the diterpene. After incubation for 16 h at 25 °C, the cells were counted under a binocular microscope.

3.3. Cytological analysis

Isolated embryonic cells were treated with diterpenes at 25 °C for 1 h. To observe the DNA synthesis (S phase), the embryonic cells were suspended in bromodeoxyuridine (BrdU) labeling medium, and incubated for 30 min at 25 °C. The cell suspensions were centrifuged at 3000 rpm for 5 min, and the pellets were washed with washing buffer and soaked in 0.045 M KCl solution for 20 min. The labeled cell suspensions were placed on glass slides, and after being left to air-dry at room temperature, the cells were fixed with acetic acid–methanol (1:3) for 1 h. The glass slides were washed three times with washing buffer and carefully dried. The cells were covered with an anti-BrdU solution and the slides were incubated for 30 min at 37 °C in a high-humidity atmosphere in a plastic-box. The glass slides were washed three times with washing buffer. Then, the cells were covered with an anti-mouse–Ig-fluorescein solution and the glass slides were incubated for 30 min at 37 °C in a humid plastic-box. The glass slides were finally washed three times with washing buffer, air-dried, and stained with 5% Giemsa in PBS for 20 min.

Table 3. Number of cells arrested in the prophase, prometaphase, and metaphase by following colchicine, kansuinin B, 20-OD-ingenol *Z*, and 20-OD-ingenol *E* treatment

Treatment		No. of prophase (%)	No. of prometaphase (%)	No. of metaphase (%)	Total no
Control					
Exp. no.	1	113 (29)	91 (23)	4 (1)	384
	2	240 (34)	180 (25)	3 (0.4)	713
	3	96 (31)	66 (22)	2 (0.7)	306
Average of % (±SD)		31 ± 2.5	23 ± 1.5	0.7 ± 0.3	
Colchicine					
Exp. no.	1	149 (43)	127 (36)	26 (7.4)	349
	2	216 (40)	235 (43)	35 (6.5)	541
	3	295 (41)	315 (44)	28 (3.9)	711
Average of % (±SD)		41 ± 1.5	41 ± 4.4	5.9 ± 1.8	
Kansuinin B					
Exp. no.	1	41 (12)	106 (32)	0 (0)	330
	2	96 (17)	189 (34)	0 (0)	574
	3	124 (23)	146 (27)	0 (0)	532
Average of % (±SD)		17 ± 5.5	31 ± 3.6	0	
20-OD-Ingenol Z					
Exp. no.	1	161 (39)	51 (12)	0 (0)	416
	2	204 (30)	148 (22)	1 (0.1)	680
	3	243 (29)	142 (17)	0 (0)	830
Average of % (±SD)		33 ± 5.5	17 ± 5.0	0.03 ± 0.06	
20-OD-Ingenol E					
Exp. no.	1	139 (36)	178 (46)	10 (2.6)	387
	2	152 (34)	187 (42)	20 (4.5)	443
	3	198 (34)	242 (42)	22 (3.8)	579
Average of % (±SD)		35 ± 1.2	43 ± 2.3	3.6 ± 0.96	

The isolated cells were treated with 0.5 μg/mL of colchicine, 50 μg/mL of kansuinin B, and 2 μg/mL of 20-OD-ingenol *Z* or 20-OD-ingenol *E* for 1 h. The cells were treated as described in Figure 3.

To observe the nuclei and the chromosomes (M phase), the embryonic cells were incubated with colchicine and the diterpenes at 25 °C for 1 h. The cell suspensions were centrifuged and the pellets were soaked in 0.045 M KCl solution for 1 h. The cells were then placed on a glass slide, air-dried, then fixed with a solution of acetic acid–methanol (1:3) for 1 h, and stained with 5% Giemsa in PBS for 30 min.

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References and notes

1. Wu, T. S.; Lin, Y. M.; Haruna, M.; Pan, D. J.; Shingu, T.; Chen, Y. P.; Hsu, H. Y.; Nakano, T.; Lee, K. H. *J. Nat. Prod.* **1991**, *54*, 823.
2. Zheng, W. F.; Cui, Z.; Zhu, Q. *Planta Med.* **1998**, *64*, 754.
3. Wang, L. Y.; Wang, N. L.; Yao, X. S.; Miyata, S.; Kitanaka, S. *J. Nat. Prod.* **2002**, *65*, 1246.
4. Wang, L. Y.; Wang, N. L.; Yao, X. S.; Miyata, S.; Kitanaka, S. *Chem. Pharm. Bull.* **2003**, *51*, 935.
5. Wang, L. Y.; Wang, N. L.; Yao, X. S.; Miyata, S.; Kitanaka, S. *J. Nat. Prod.* **2003**, *66*, 630.
6. Miyata, S.; Wang, L. Y.; Wang, N. L.; Yao, X. S.; Kitanaka, S. *Cell. Biol. Int.* **2004**, *28*, 179.
7. Pagano, M.; Pepperkok, R.; Verde, F.; Ansorge, W.; Draetta, G. *EMBO J.* **1992**, *11*, 961.
8. Guadagno, T. M.; Ohtsubo, M.; Roberts, J. M.; Assoian, R. K. *Science* **1993**, *262*, 1572.
9. Draetta, G.; Beach, D. *J. Cell. Sci.* **1989**, *12*, 21.
10. Brandeis, M.; Hunt, T. *EMBO J.* **1996**, *15*, 5280.
11. Hunt, T.; Luca, F. C. *J. Cell. Biol.* **1992**, *116*, 707.
12. Blow, J. J.; Nurse, P. A. *Cell* **1990**, *62*, 855.
13. Fang, F.; Newport, J. W. *Cell* **1991**, *66*, 855.
14. Newport, J.; Kirschner, M. *Cell* **1982**, *30*, 687.
15. Newport, J.; Kirschner, M. *Cell* **1982**, *30*, 675.
16. Nigg, E. A. *Trends Cell. Biol.* **1993**, *3*, 296.
17. Minshull, J.; Pines, J.; Golsteyn, R.; Standart, N.; Mackie, S.; Colman, A.; Blow, J.; Ruderman, J. V.; Wu, M.; Hunt, T. *J. Cell. Sci. Suppl.* **1989**, *12*, 77.
18. Adachi, Y.; Luke, M.; Laemmli, U. K. *Cell* **1991**, *64*, 137.
19. Hirano, T.; Mitchison, T. J. *Cell* **1994**, *79*, 449.
20. Saitoh, N.; Goldberg, I. G.; Wood, E. R.; Earnshaw, W. C. *J. Cell. Biol.* **1994**, *127*, 303–318.
21. Heck, M. M. *Cell* **1997**, *91*, 5–8.