Lawrence Berkeley National Laboratory
Recent Work

Title
Two protein 4.1 domains essential for mitotic spindle and aster microtubule dynamics and organization in vitro

Permalink
https://escholarship.org/uc/item/6xk2f355

Journal
Journal of Biological Chemistry, 279

Authors
Krauss, Sharon Wald
Lee, Goria
Chasis, Joel Anne
et al.

Publication Date
2004-05-13
JBC proofs: M402813200 article 2724 ready for download

Dear Dr. S. Krauss:

Email Address: your e-mail address
Password: ----

To view your Journal of Biological Chemistry article, please refer to this URL address
http://rapidproof.cadmus.com/RapidProof/retrieval/index.jsp

You will need to have Adobe Acrobat(R) Reader software to read these files. This is free software and is available for user downloading at http://www.adobe.com/products/acrobat/readstep.html.

Based on the number of copies requested please refer to Reprint Quantity Order Chart to determine the cost of the Reprints. To assist you with the calculation of Publication Fees here are the estimates:

Reprint number: 1160950
Page charges ($75 per journal page): $ 600
Halftones, $20.00 each, black & white: $ 20
*Heavy Paper, $120.00 per article: $ 120
Color Figures, $300.00 each: $ 1500

*Mandatory if color figures, optional if black & white figures

After printing the PDF file (use normal quality), please read the page proofs carefully and:
1) indicate changes or corrections in the margin of the page proofs;
2) answer all queries (footnotes A,B,C, etc.) on the query sheet;
3) proofread any tables and equations carefully;
4) check that any Greek, especially mu, has translated correctly.

The proofs are representative of how your article will look online. Within 24 hours, please express mail the following to the address given below (WE CANNOT ACCEPT FAXES OF PROOFS):
1) original PDF set of page proofs including query sheet with answers (by overnight mail)
2) IF figure corrections are needed, please print quality hard copy for the figures (we CANNOT accept figures on disk at this time for corrections),
3) Notice to Author form (cannot print without this)
4) Signed Copyright Assignment form (cannot print without this)
5) Reprint Order form (including the price sheet).

PLEASE INCLUDE YOUR ARTICLE NO. ( 2724 ) WITH ALL CORRESPONDENCE.

Attn: Amy Strasser
Journal of Biological Chemistry
940 Elkridge Landing Road
Linthicum, MD 21090-2908
strassera@cadmus.com
Tel: 410-691-6990
Fax: 410-684-2790
# Proofreader’s Marks

| MARK | EXPLANATION | EXAMPLE |
|------|-------------|---------|
| ~    | TAKE OUT CHARACTER INDICATED | ~ Your proof. |
| ^    | LEFT OUT, INSERT | ^ Your proof. |
| #    | INSERT SPACE | # Your proof. |
| 9    | TURN INVERTED LETTER | 9 Your proof. |
| ×    | BROKEN LETTER | × Your proof. |
| !@#  | EVEN SPACE | !@# A good proof. |
| 0    | CLOSE UP: NO SPACE | 0 Your proof. |
| tr   | TRANSPOSE | tr A proves good |
| wf   | WRONG FONT | wf Your proof. |
| lc   | LOWER CASE | lc Your proof. |
| caps | CAPITALS | caps Your proof. |
| italic | ITALIC | italic Your proof. |
| rom | ROMAN, NON ITALIC | rom Your proof. |
| bf   | BOLD FACE | bf Your proof. |
| ...... | LET IT STAND | ..... Your proof. |
| out sc | DELETE, SEE COPY | out sc She Your proof. |
| spell out | SPELL OUT | spell out Queen (Eliz.) |
| §    | START PARAGRAPH | § read. Your |
| no § | NO PARAGRAPHS: RUN IN | no § marked Your proof. |
| 1    | LOWER | 1 (Your proof.) |
| 1    | RAISE | 1 Your proof.1 |
| □    | MOVE LEFT | □ Your proof. |
| □    | MOVE RIGHT | □ Your proof. |
|  || | ALIGN TYPE | Three dogs. Two horses |
|  —  | STRAIGHTEN LINE | — Your proof. |
| ○    | INSERT PERIOD | ○ Your proof. |
| ;/   | INSERT COMMA | ;/ Your proof. |
| ;/   | INSERT COLON | ;/ Your proof. |
| ;/   | INSERT SEMICOLON | ;/ Your proof. |
| ∨    | INSERT APOSTROPHE | ∨ Your proof. |
| ≠/   | INSERT QUOTATION MARKS | ≠/ Marked it proof |
| =/   | INSERT HYPHEN | =/ A proofmark. |
| !    | INSERT EXCLAMATION MARK | ! Prove it |
| ?    | INSERT QUESTION MARK | ? Is it right |
| ?    | QUERY FOR AUTHOR | ? Your proof read by |
| c/j | INSERT BRACKETS | c/j The Smith girl |
| c/j | INSERT PARENTHESIS | c/j Your proof |
| ∕    | INSERT 1-EM DASH | ∕ Your proof. |
| □    | INDENT 1 EM | □ Your proof. |
| □    | INDENT 2 EMS | □ Your proof. |
| □□   | INDENT 3 EMS | □□ Your proof. |
Reprint order forms and purchase orders or prepayments must be received 2 weeks before Publication either by mail or by fax at 410-820-9765. It is the policy of The Journal of Biological Chemistry to issue one invoice per order.

Author Name _____________________________________________________________________________________
Title of Article _______________________________________________________________________________________________
**No. of Pages: **_________ **Manuscript No. **M402813200 **Reprint No.: **1160950 Pub Date: ____________

Color in Article? Yes / No (Please Circle)
Category: _______________________________
Color in Article?    Yes   /   No       (Please Circle)

Please include the journal name and reprint number or manuscript number on your purchase order or other correspondence.

** IMPORTANT

The following information MUST be transferred from the Notification Email to this Reprint Order Form.
Manuscript No.
Reprint No.
No. of Pages
Page Charges ($75 per journal page)
Halftone Charges ($20 each, black and white)
Color Figure Charges ($300 each)
Heavy Paper Charge ($120 per article)

Please note:
- Heavy Paper Charge is mandatory if the article contains color figures and optional if it does not.
- A completed Reprint Order Form must be submitted to Cadmus Reprints regardless of whether reprints are ordered.

Minimum order is 100 copies. Orders are accepted in multiples of 100 copies only. For articles longer than 20 pages, please call Cadmus Reprints at 800-407-9190.

Prepayment or a signed institutional purchase order is required to process your order. This form may be used as a proforma invoice. Please send your order form and purchase order or payment made payable to:
ASBMB
P.O. Box 630485
Baltimore, MD 21263-0485
FEIN 52-6050165

If sending by registered mail. Send to ASBMB- Reprints, 940 Elkridge Landing Road, Linthicum, MD 21090-2908. Please call 800-407-9190 or 410-819-3993 or fax 410-820-9765 or e-mail WolfJ@cadmus.com if you have any questions.

Enclosed:  Personal Check: ________
Institutional Purchase Order: ________
Credit Card Payment: ________

Checks must be paid in U.S. Dollars and drawn on a U.S. Bank

Please see page 2 of 2 for reprint costs and shipping instructions.

**Reprint Costs
_____ number of reprints ordered $__________
* Add color in reprints: $50 per 100 copies $__________
Taxes $__________
(Add appropriate sales tax for Virginia, Maryland, Pennsylvania and the District of Columbia or 7% Canadian GST to the reprints if your order is to be shipped to these locations.)
Add $32.00 for each additional ship location $__________
* Call for quote if 600 or more color reprints are wanted.

**Publication Fees
**Page Charges, $75 per journal page $600.00
**Halftones, $20 each $20.00
**Heavy Paper, $120 per article $120.00
**Color Figures, $300 each $1600

Total Amount Due $__________
(Reprint Cost & Publication Fees)

**Ordering Details

Invoice Address
Name _____________________________________________________________________________________
Institution _______________________________________________________________________________________________
Department _______________________________________________________________________________________________
Street _______________________________________________________________________________________________
City _____________ State _____ Zip _________
Country _______________________________________________________________________________________________
Phone __________________   Fax __________________
E-mail Address _______________________________________________________________________________________________
Purchase Order No. _______________________________________________________________________________________________

Credit Card Payment Details

Credit Card:  ____ VISA  ____ Am. Exp.  ____ MasterCard
Card Number __________________
Expiration Date __________________
Signature __________________

Cadmus will process credit cards and CADMUS JOURNAL SERVICES will appear on the credit card statement.

Signature __________________
Date __________________
Signature is required. By signing this form, the author agrees to accept the responsibility for the payment of reprints and/or all charges described in this document.

Page 1 of 2  JW-1003
**SHIPPING INSTRUCTIONS/INFORMATION**

**Shipping Address** (cannot ship to a P.O. Box)

Name _________________________________________
Institution______________________________________
Street _________________________________________
City ______________  State ______  Zip ___________
Country________________________________________
Quantity___________________  Fax ________________
Phone:  Day ________________  Evening ____________

**Additional Shipping Address** (cannot ship to a P.O. Box)

Name _________________________________________
Institution______________________________________
Street _________________________________________
City ______________  State ______  Zip ___________
Country________________________________________
Quantity___________________  Fax ________________
Phone:  Day ________________  Evening ____________

* Add $32.00 for each additional shipping address

---

**Shipping and Delivery:** UPS ground within the United States (1-5 days delivery) is included in the reprint prices, except for orders in excess of 1000 copies. Orders shipped to authors outside the United States are mailed via an expedited air service. Your order will be shipped within 2 weeks of the journal print date.

### Black and White Reprint Prices : Domestic Copies (USA Only)

| # of Pages | 100  | 200  | 300  | 400  | 500  | 600  | 700  | 800  | 900  | 1000  | Add'l 100’s |
|------------|------|------|------|------|------|------|------|------|------|--------|-------------|
| 2          | $227 | $235 | $245 | $257 | $273 | $287 | $300 | $315 | $329 | $348   | $14         |
| 4          | $264 | $276 | $285 | $297 | $313 | $327 | $343 | $359 | $373 | $392   | $16         |
| 6          | $343 | $454 | $481 | $511 | $540 | $566 | $598 | $631 | $658 | $689   | $31         |
| 8          | $471 | $492 | $518 | $549 | $577 | $604 | $635 | $669 | $696 | $726   | $31         |
| 10         | $624 | $664 | $710 | $756 | $804 | $850 | $896 | $941 | $983 | $1,035 | $45        |
| 12         | $661 | $702 | $748 | $793 | $842 | $887 | $933 | $979 | $1,021| $1,072 | $45        |
| 14         | $801 | $870 | $931 | $995 | $1,056| $1,114| $1,177| $1,268| $1,336| $1,403 | $60        |
| 16         | $849 | $908 | $969 | $1,033| $1,094| $1,152| $1,215| $1,306| $1,374| $1,440 | $60        |
| 18         | $1,005| $1,083| $1,164| $1,242| $1,316| $1,422| $1,503| $1,591| $1,677| $1,758 | $71        |
| 20         | $1,043| $1,121| $1,202| $1,280| $1,353 | $1,460| $1,541| $1,629| $1,714| $1,795 | $71        |

### Black and White Reprint Prices : International Copies (including Canada and Mexico)

| # of Pages | 100  | 200  | 300  | 400  | 500  | 600  | 700  | 800  | 900  | 1000  | Add'l 100’s |
|------------|------|------|------|------|------|------|------|------|------|--------|-------------|
| 2          | $244 | $252 | $266 | $282 | $307 | $325 | $343 | $363 | $385 | $408   | $18         |
| 4          | $281 | $301 | $323 | $344 | $373 | $395 | $424 | $448 | $475 | $501   | $23         |
| 6          | $459 | $501 | $548 | $599 | $649 | $695 | $746 | $800 | $848 | $899   | $50         |
| 8          | $496 | $539 | $585 | $637 | $686 | $733 | $783 | $838 | $886 | $936   | $50         |
| 10         | $662 | $731 | $810 | $884 | $964 | $1,040| $1,114| $1,192| $1,262| $1,345 | $76         |
| 12         | $699 | $769 | $848 | $921 | $1,002| $1,077| $1,151| $1,230| $1,300| $1,382 | $76         |
| 14         | $849 | $959 | $1,060| $1,164| $1,266| $1,365| $1,467| $1,599| $1,707| $1,814 | $100        |
| 16         | $897 | $997 | $1,098| $1,202| $1,304| $1,403| $1,505| $1,637| $1,745| $1,851 | $100        |
| 18         | $1,065| $1,192| $1,324| $1,452| $1,575 |$1,732 |$1,863 |$1,998 |$2,137 |$2,268 |$121        |
| 20         | $1,103| $1,230| $1,362| $1,490| $1,612 |$1,770 |$1,901 |$2,036 |$2,174 |$2,305 |$121        |
NOTICE TO AUTHORS

To avoid delay in publication, please provide complete information to the following questions.

Category
The Table of Contents is arranged by Category. Please indicate which of the following is the appropriate Category for your paper.

- 1. DNA: Replication, Repair, and Recombination
- 2. Genes: Structure and Regulation
- 3. RNA: Structure Metabolism, and Catalysis
- 4. Protein Syntheses, Post-translation Modification, and Degradation
- 5. Genomics, Proteomics, and Bioinformatics
- 6. Protein Structure and Folding
- 7. Enzyme Catalysis and Regulation
- 8. Metabolism and Bioenergetics
- 9. Glycobiology and Extracellular Matrices
- 10. Lipids and Lipoproteins
- 11. Membrane Transport, Structure, Function, and Biogenesis
- 12. Mechanisms and Signal Transduction
- 13. Molecular Basis of Cell and Developmental Biology

Authors of Accelerated Publication papers: If your paper exceeds 4 pages in length and you choose to run it as a regular article, please indicate category.

Reprints
Please indicate the number of reprints ordered: ________________

Paper Selection

No Color
We recommend that articles with gels and electron micrographs be printed on heavy paper for higher quality reproduction. Cost for heavy paper is $120: cost for each halftone is $20 regardless of paper choice. For reproduction of figure(s) ________________ on heavy paper, total cost would be $

Please circle your choice of paper. If you have no halftones, your paper will automatically be printed on regular stock paper.

| HEAVY PAPER | REGULAR PAPER |

Color
Your article will automatically be printed on heavy paper.

Revised Page Proofs
Revised page proofs can be faxed to you if you have a specific concern regarding the makeup of your paper or if there are extensive alterations. Please note that revised page proofs are used for confirmation purposes only and are not considered as a second opportunity for corrections.

☐ I do not need revised page proofs. Please expedite publication after incorporating any changes I have indicated.
☐ Please fax page proofs.

PLEASE SIGN COPYRIGHT NOTICE.
PLEASE RETURN PAGE PROOFS AND ALL OTHER CORRESPONDENCE WITHIN 48 HOURS BY EXPRESS MAIL, NEXT DAY DELIVERY.

THANK YOU.
---COPYRIGHT ASSIGNMENT---

Papers cannot be published unless a signature is on file. Please return this form with your page proofs.

To: American Society for Biochemistry and Molecular Biology, Inc. (ASBMB)

I/We hereby confirm that

a) The ASBMB shall, in consideration of publication, become entitled to the work copyright and translation rights therein and that I/we assign all copyrights in the paper to ASBMB for publication in printed and electronic forms.

b) For U.S. GOVERNMENT EMPLOYEES: This work was done in my capacity as a U.S. government employee; the above assignment applies only to the extent allowable by law.

Signed: The signing author must indicate that consent is held from each co-author for copyright to be assigned to ASBMB.

Date: ____________________________

Signed: __________________________________________________________________________

☐ I hold consent from each co-author for copyright to be assigned to ASBMB.
COLOR ARTICLES ONLY

We CANNOT publish your article until we receive approval of the reproduction quality for your color figure(s). Please view your color images on the computer screen and fax or e-mail your approval to me within 24 hours, referring to the article number.

Thank you,

Dan Scott

Journal of Biological Chemistry
940 Elkridge Landing Road
Linthicum, MD
21090-2908
Tel: 410-691-6256
Fax: 410-684-2790
E-mail: scottd@cadmus.com

P.S. When you return the page proofs, please be sure to indicate the total number of reprints desired. It is also important to return your reprint order form as quickly as possible to make sure the color reprints are ordered promptly.
I would like to subscribe to JBC at the rate circled.

| Option                | U.S.* | Non U.S.* |
|-----------------------|-------|-----------|
| Print only            | $2,050| $2,550    |
| Online only           | $1,950| $1,950    |
| Print + Online        | $4,000| $4,500    |

Is this a NEW subscription or a RENEWAL? If a renewal, list your current customer number, if known ____________.

* Canadian subscriptions: please include GST number if available. The GST of 7% must be added to the U.S. price. Maryland residents add 5% sales tax or provide tax exempt ID. **Contact the subscription office for expedited shipping prices.**

**Payment Options:**
- **VISA**
- **MasterCard**
- **American Express**

Card # _______________________________ Exp. Date___________________
Cardholder’s Signature: ____________________________________________
Cardholder’s Name: (print/type)______________________________________
Email: ____________________________Tel:______________________________

- Check or Money Order. **US currency only. Drawn on US bank.**

**Ship to Address:**
Name ___________________________________________________________
Organization ______________________________________________________
Address __________________________________________________________
City/State/Zip-Postal Code/Country __________________________________
Email (required): ____________________________________________Tel:____________ Fax:____________

**FAX ORDER TO:** 205-995-1588
**PHONE ORDER TO:** 1-800-633-4931 OR 205-995-1567
**MAIL ORDER TO:** Journal of Biological Chemistry (JBC)
PO Box 830399
Birmingham, AL 35283-0399

**QUESTIONS? CALL CUSTOMER SERVICE TOLL FREE:** 1-800-633-4931, OR 205-995-1567

**VISIT OUR WEBSITE:** www.jbc.org
Send comments or questions to: jbc@asbmb.faseb.org
Molecular and Cellular Proteomics will have an emphasis placed on determining how the presence or absence of proteins affects biological responses and how the interaction of proteins with relevant cellular partners allows them to function. Articles utilizing or advancing protein identification technology — such as multi-dimensional electrophoresis and/or mass spectrometry — protein and nucleic acid arrays, and computational assessments will be particularly appropriate.

• In addition to manuscripts describing research advances in proteomics, articles concerning technological advances will also be accepted. In addition, MCP will publish large data sets as either appendices to regular manuscripts or as stand alone contributions. The latter must include a summary, not to exceed two printed pages, describing the germane points and importance of the information. The data sets themselves (either as appendices or as separate articles) will appear only in the on-line version. A letter of intent describing the extent and format of this supplemental material must precede submission of the manuscript.

• Electronic Manuscript Submission — Manuscript submission, review, and initial appearance will all be accomplished electronically (the e-version will be published as a member of the HighWire consortium).

• Immediate Publication — All papers accepted for publication will appear immediately as a Paper in Press.

• Printed Monthly — The print version will appear on a monthly basis (without supplemental information).
Opening Lecture
First Annual Herbert Tabor/Journal of Biological Chemistry Lectureship
Robert J. Lefkowitz, HHMI, Duke University Medical Center

Organized by:
John D. Scott, HHMI, Vollum Institute; Alexandra C. Newton, UCSD; Julio Celis, Danish Cancer Society, and the 2004 ASBMB Program Planning Committee

Cellular Organization and Dynamics
Organizer: Harald A. Stenmark, Norwegian Rad. Hosp.

Genomics, Proteomics and Bioinformatics
Organizers: Charlie Boone, Univ. of Toronto and Michael Snyder, Yale Univ.

Integration of Signaling Mechanisms
Organizer: Kjetil Tasken, Univ. of Oslo, Norway

Molecular and Cellular Biology of Lipids
Organizer: Dennis Vance, Univ. of Alberta

Molecular Recognition and Catalysis
Organizer: Jack E. Dixon, UCSD

Protein Modifications and Turnover
Organizer: William J. Lennarz, SUNY at Stony Brook

Protein Structure, Catalysis and Dynamics
Organizer: Susan Taylor, UCSD

Regulation of Gene Expression and Chromosome Transactions
Organizer: Joan W. Conaway, Stowers Inst. for Med. Res.

Signaling Pathways in Disease
Organizers: Alexandra Newton, UCSD and John D. Scott, HHMI, Vollum Inst.

The Future of Education and Professional Development in the Molecular Life Sciences
Organizer: J. Ellis Bell, Univ. of Richmond

For further information:
ASBMB
9650 Rockville Pike
Bethesda, MD 20814
Tel: 301-634-7145
Fax: 301-634-7126
Email: asbmb@asbmb.faseb.org
http://www.asbmb.org

www.faseb.org/meetings/asmb04
Abstract Deadline: February 11, 2004

American Society for Biochemistry and Molecular Biology Annual Meeting
and 8th IUBMB Conference

June 12 – 16
Boston, MA

IUBMB/ASBMB 2004
“A Molecular Exploration of the Cell”
Two Protein 4.1 Domains Essential for Mitotic Spindle and Aster Microtubule Dynamics and Organization in Vitro*

Received for publication, March 12, 2004, and in revised form, April 16, 2004
Published, JBC Papers in Press, April 21, 2004, DOI 10.1074/jbc.M402813200

Sharon Wald Krauss‡‡, Gloria Lee†, Joel Anne Chasis†, Narla Mohandas†, and Rebecca Heald¶

From the §Lawrence Berkeley National Laboratory, Life Sciences Division, University of California, Berkeley, California 94720, the ¶New York Blood Center, New York, New York 10021, and the ¶¶Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

Multifunctional structural proteins belonging to the 4.1 family are components of nuclei, spindles, and centrosomes in vertebrate cells. Here we report that 4.1 is critical for spindle assembly and the formation of centrosome-nucleated and motor-dependent self-organized microtubule asters in metaphase-arrested Xenopus egg extracts. Immunodepletion of 4.1 disrupted microtubule arrays and mislocalized the spindle pole protein NuMA. Remarkably, assembly was completely rescued by supplementation with a recombinant 4.1R isoform. We identified two 4.1 domains critical for its function in microtubule polymerization and organization utilizing dominant negative peptides. The 4.1 spectrin-actin binding domain or NuMA binding C-terminal domain peptides caused morphologically disorganized structures. Control peptides with low homology or variant spectrin-actin binding domain peptides that were incapable of binding actin had no deleterious effects. Unexpectedly, the addition of C-terminal domain peptides with reduced NuMA binding caused severe microtubule destabilization in extracts, dramatically inhibiting aster and spindle assembly and also depolymerizing preformed structures. However, the mutant C-terminal peptides did not directly inhibit or destabilize microtubule polymerization from pure tubulin in a microtubule pelleting assay. Our data showing that 4.1 is a crucial factor for assembly and maintenance of mitotic spindles and self-organized and centrosome-nucleated microtubule asters indicates that 4.1 is involved in regulating both microtubule dynamics and organization. These investigations underscore an important functional context for protein 4.1 in microtubule morphogenesis and highlight a previously unappreciated role for 4.1 in cell division.

Protein 4.1, formerly characterized solely as a crucial membrane skeletal protein in mature red cells, is now also recognized to be an important multifunctional structural protein family in nucleated cells. Although protein 4.1 can be plasma membrane-associated in nucleated cells, it also is detected at diverse and interesting subcellular locations during the cell cycle. Protein 4.1 isoforms localize within the nucleus and at centrosomes during interphase, at spindle poles during mitosis, in perichromatin at anaphase, and in the midbody at telophase (1–3). Whereas mature red cells express only 80-kDa 4.1, the complex subcellular localization patterns of 4.1 in mammalian cells likely result from expression of several 4.1 isoforms, post-translational modifications and expression of multiple 4.1-related genes (4.1R, G, B, and N) (4–14).

Beyond the characterization of its localization, the current challenge is to decipher functions of 4.1 in various subcellular structures. Although the list remains incomplete, a number of protein 4.1 binding partners have been identified to interact with a specific 4.1 domain in red cells and/or nucleated cells, providing some clues as to potential 4.1 functions (see Fig. 1). Prototypical protein 4.1 (R, red cell) contains several functional domains. An N-terminal extension present only in some isoforms in nucleated cells has been found to interact with the centrosomal protein CPAP (centrosome protein-4.1 associated protein) (15). The FERM (4.1/ezrin, radixin/moesin) domain interacts with plasma membrane-binding proteins and was recently discovered to also contain a microtubule binding site (16). The spectrin-actin binding domain (SABD) (17) is capable of forming ternary complexes with spectrin and actin. The C-terminal domain is of particular interest, because it has been found to interact with NuMA (nuclear mitotic apparatus protein) (17). These observations suggest that 4.1 plays diverse roles within the cytoskeleton.

Recently we showed (18, 19) that 4.1 is essential for the assembly of functional nuclei in interphase Xenopus egg extracts and requires its capacity to bind actin, which is found closely associated with 4.1 on nuclear filaments in mammalian cells. This latter observation was recently confirmed and extended in a study (20) that characterized an extensive system of nuclear pore-linked filaments in Xenopus oocytes that contain actin and 4.1 epitopes. Here we provide evidence that 4.1 is also essential during mitosis in Xenopus egg extracts for proper polymerization and organization of the microtubule cytoskeleton.

During interphase, centrosomes nucleate and organize a radial array of microtubules. We characterized protein 4.1 previously as an integral centrosome component, resisting vigorous salt/detergent extraction and present in centrosomes independent of microtubules. By immunofluorescence and cell whole mount electron microscopy 4.1 epitopes localized on centrioles, in the pericentriolar matrix, and on the fibers connecting the centriolar pair (1). During mitosis duplicated centrosomes become the poles of the mitotic spindle apparatus, contributing to the organization of microtubules into a bipolar array with their minus ends focused at the poles and their plus ends interacting with chromosomes and overlapping within the center of the

* This work was supported by Grants DK59079 (to S. W. K.), GM057839 (to R. H.), and DK32094 (to S. W. K., J. A. C., and N. M.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: University of California, Lawrence Berkeley National Lab, 1 Cyclotron Rd, MS 74-157, Berkeley, CA 94720. Tel.: 510-486-4073; Fax: 510-486-6746; E-mail: sakrauss@lbl.gov.

This paper is available online at http://www.jbc.org

†‡ The abbreviations used are: SABD, spectrin-actin binding domain; NuMA, nuclear mitotic apparatus protein; PIPES, 1,4-piperazinediethanesulfonic acid; R, red cell.

†‡ The abbreviations used are: SABD, spectrin-actin binding domain; NuMA, nuclear mitotic apparatus protein; PIPES, 1,4-piperazinediethanesulfonic acid; R, red cell.
spindle. Ultimately responsible for accurate chromosome segregation, spindle function is also dependent on a variety of microtubule-based motor proteins including dynein and kinesin-related proteins that cross-link and sort microtubules according to their structural polarity, and mediate chromosome interactions within the spindle. Proper spindle pole organization is known to depend on the function of NuMA, which interacts with dynein and contributes to microtubule minus-end cross-linking to maintain spindle pole structure (as reviewed in Ref. 21) (22–26). Taken together, the observations that protein 4.1 binds to NuMA and localizes to centrosomes and mitotic spindle poles raise the intriguing hypothesis that protein 4.1 is involved in cell division.

In this report we used Xenopus egg extracts, a powerful system for in vitro dissection of mitotic microtubule assembly, organization, and function. Xenopus eggs are laid arrested in the metaphase of meiosis II by cytostatic factor, until fertilization triggers a calcium wave promoting entry into the first mitotic cell cycle. Extracts prepared from unfertilized eggs maintain the cytostatic factor metaphase arrest. Mitotic microtubule asters analogous to spindle poles can be assembled in vitro with addition of purified centrosomal proteins. Some asters were assembled for 15 min at 20 °C for assembly of spindles, centrosomes, or self-organized asters as indicated. Although a range of 1–8 μg was tested for each peptide, the data presented are from experiments using 8 μg of the indicated peptide.

**Microtubule Co-pelleting Assay—**Solutions of 4.1 peptides and 18 μM of tubulin and 1 mM GTP in BRB80 were preincubated with centrifuged X. laevis egg extract to form a 1:100 rotor for 15 min at 40,000 rpm at 4 °C. Reactions containing 1 mM GTP, 35 μM tubulin, 35 μM peptide were mixed on ice, and 5% Me2SO was added (final concentration 5%). The reaction was incubated for 30 min at 37 °C, layered onto 40% BRB80 sucrose cushions, and microtubules were pelleted at 40,000 rpm for 20 min in a TL-100 rotor. Equivalent amounts of supernatant and pellet were analyzed by Western blotting using a 15% SDS-polyacrylamide gel. By this assay, inhibitors of microtubule formation show tubulin remaining in the supernatant (34).

**Immunodepletion and Rescue—**For 4.1 depletion from Xenopus extracts, protein G-coupled magnetic beads (Dynal) from 100 μl of slurry were mixed with 15 μg of 4.1R SABD, C-terminal domain IgGs or non-immune rabbit IgG for 4 h at 4 °C, the beads were washed twice with 0.1 M sodium phosphate buffer, pH 7.0 (57.7% Na2HPO4 and 42.3% NaH2PO4, w/v), and three times with XB buffer (20) then divided into three aliquots. Extract (100 μl) was successively depleted three times by rotation with IgG-coupled beads at 4 °C for 1 h. beads were collected magnetically, and extract was used for the assembly of spindles, centrosome asters, or self-organized asters. Extract depletion was estimated by densitometry of Western blots using an Alpha Imager 2200 and software. In rescue experiments, 1–9 μg of purified bacterially expressed 4.1R 4.1 was added to 20 μl reactions and incubated on ice for 10 min prior to the initiation of assembly. Reactions in three independent experiments were sampled during 15–45 min incubation periods for the assembly of spindles, centrosomes, or asters. The experiment presented was performed in parallel using the same depleted extract as described (19).

**RESULTS**

**4.1 Localizes to Mitotic Spindles, Centrosome Asters, and Self-organized Asters Reconstituted in Xenopus Egg Extracts—**Previous reports (1, 2, 16, 17) using a variety of mammalian cells established that 4.1 is localized to centrosomes and mitotic spindle poles. As the Xenopus laevis 4.1 sequence has many highly homologous regions relative to mammalian family members, including the SABD and C-terminal domains (Fig. 1) (19), we anticipated a similar 4.1 localization in Xenopus. Furthermore, 4.1 function was shown to be conserved between the frog and mammals in studies reconstituting properties of 4.1-deficient human erythrocyte membranes using a recombinant Xenopus 4.1 domain (35). We first verified that 4.1 epitopes could be detected in centrosomes and mitotic spindles of cultured Xenopus fibroblasts (data not shown). Next we incubated cytoplasmic extracts from Xenopus eggs with an antibody against NuMA and the antibody against protein 4.1 of KE37 centrosomes at 2 °C for 30 min, fixed in 3% paraformaldehyde, and permeabilized with 1% Triton X-100 and spun onto coverslips through a cushion of BRB80 containing 15% glycerol. Immunofluorescence microscopy showed that protein 4.1 is localized to centrosomes and mitotic spindles of cultured Xenopus fibroblasts (data not shown).

**EXPERIMENTAL PROCEDURES**

**Materials—**Expression vectors for His-tagged proteins were either pMW172 (the gift of Dr. M. Way, European Molecular Biology Laboratory, Heidelberg, Germany) or PET 28 (Novagen). The antibody against NuMA was a very generous gift of Dr. A. Merdes (University of Edinburgh, Scotland). IgG against 4.1R SABD and 4.1R C-terminal domain were described (2). Fluorescent secondary antibodies were from Molecular Probes. Bovine brain tubulin was prepared according to Ashford et al. (28).

**Xenopus Extracts and Assembly Reactions—**10,000 × g cytoplasmic Xenopus egg extracts and demembranated sperm nuclei were prepared as described (29). For spindle assembly, demembranated Xenopus sperm were added to 20 μl of egg extract on ice with 0.2 mg/ml Texas Red-labeled tubulin, reactions incubated at 20 °C for 30–45 min, diluted with BRB80 (80 mM PIPES, 2 mM MgCl2, 1 mM EGTA, pH 6.8) containing 30% glycerol and 1% Triton X-100 and spun through BRB80 cushions with 40% Me2SO on coverslips (20). Self-organized microtubule asters were assembled by addition of Me2SO (final concentration 5%) to the egg extract and incubation for 15 min at 20 °C (31). Centrosome asters were assembled for 15 min at 20 °C after the addition of 1 μl of KE37 centrosomes at 2 × 106/ml prepared according to Moudjou and Bornens (32), to 20 μl of extract that had been centrifuged for 30 min at 60,000 rpm in a TLA 100.3 rotor. Centrosome or self-organized aster reactions were diluted with BRB80 containing 15% glycerol and 1% Triton X-100 and spun onto coverslips through a cushion of BRB80 containing 30% glycerol.

**Indirect Immunofluorescence—**In vitro assembled structures on coverslips were fixed in –20 °C MeOH and probed by immunofluorescence as described (33). The coupled secondary antibodies were from SABD IgG, 5 μg/ml; C-terminal domain IgG, 10 μg/ml; anti-NuMA, 1:50 dilution. Secondary antibodies were used at a 1:100 dilution. Samples probed with equal amounts of control non-immune IgG or without primary antibody or sera showed no fluorescent patterns. Images were captured using a Nikon Eclipse 2000 microscope equipped with a CCD camera and processed using Adobe Photoshop. Under the imaging conditions used, the limits of resolution of overlap between two fluorophores (e.g. superimposition of red and green signals to generate yellow coloration) was estimated to be –300 nm.

**Expression and Purification of His-tagged Proteins—**Protein 4.1-related peptides were expressed and purified as described (19). Assays with His6 peptides (1–8 μg) were preincubated on ice for 10 min then incubated at 20 °C for assembly of spindles, centrosomes, or self-organized asters as indicated. Although a range of 1–8 μg was tested for each peptide, the data presented are from experiments using 8 μg of the indicated peptide.
Protein 4.1 Domains in Spindle and Centrosome Assembly

Fig. 1. Domain organization of protein 4.1 and interacting proteins. A, a schematic map of 4.1R indicating functional interacting domains. Exon numbers appear below the bar with asterisks indicating alternatively spliced exons. Arrows indicate translation initiation sites. Isoforms initiated at AUG1 include the N-terminal extension (exons 2–4). The membrane binding 30-kDa/FERM domain extends from exon 4 to 12. In this report, the spectrin-actin binding domain (SABD) refers to amino acid sequences from exons 16 and partial 17, whereas the C-terminal domain denotes peptides from exons 20 to 21. B, proteins known to interact with protein 4.1 domains. The proteins are listed under and color-coded corresponding to their 4.1 interaction domain. CPAP refers to centrosome protein-4.1-associated protein (15).

Each reaction, microtubules were stained by the addition of trace amounts of rhodamine-labeled tubulin. When each of these structures was probed by immunofluorescence microscopy using affinity-purified antibodies against the 4.1R SABD or C-terminal domain or against the 80-kDa 4.1R, strong 4.1 signals were concentrated at the minus ends of microtubules focused at the spindle poles and also in the center of both centrosome and self-assembled asters (Fig. 2).

Immunodepletion of 4.1 Compromises Assembly of Microtubule-Based Structures and Can Be Rescued by Supplementation with Recombinant 4.1R—To test whether the protein 4.1 itself is essential for the assembly of spindles, centrosome asters, and self-organized asters, we depleted 4.1 from Xenopus extracts using 4.1 domain-specific affinity-purified IgGs bound to protein G magnetic beads. Previously (19) we showed by Western blotting that Xenopus extracts contain protein bands from 47 to 110 kDa detected by SABD and C-terminal domain IgGs, which were reduced by 50–100% after three rounds of antibody depletion. Using 4.1-immunodepleted extracts, we evaluated the morphology of spindles, centrosomes, and self-organized microtubule asters, and as an additional indicator of structural integrity we localized the 4.1 binding partner/spindle pole protein NuMA by immunofluorescence. In controls, NuMA largely localized with 4.1 epitopes in a tight focus at the spindle poles, and in the centers of centrosome and self-organized microtubule asters (Figs. 2 and 3).

Normal spindles, centrosome asters, and self-organized asters assembled in egg extracts mock-depleted with nonspecific IgG and protein G beads (Fig. 3A). In contrast, there was a dramatic morphological disruption of all microtubule structures assembled in extracts depleted with either 4.1 domain-specific SABD or C-terminal domain IgGs. In spindles assembled in either depleted extract, chromosomes were not aligned equidistant from the poles but were looped out of the spindle midzone (Fig. 3A). Spindles formed in SABD-depleted extracts most often were multipolar, whereas those from C-terminal domain-depleted extracts generally had large unfocused poles. Centrosome asters were disorganized microtubule arrays without an obvious focal center revealed by NuMA staining (Fig. 3B). In SABD-depleted extracts, centrosomes asters often contained multiple small NuMA foci radiating several bundles of microtubules. Similarly, self-organized asters assembled in either the C-terminal domain or SABD-depleted extracts had disorganized microtubules, and NuMA was mislocalized (Fig. 3C). Therefore, depleting SABD- and C-terminal domain-containing 4.1 proteins disrupted spindle, centrosome aster, and self-organized microtubule aster assembly.

The aberrant reconstitution of microtubule structures observed in depleted extracts could result either from loss of 4.1 function itself or from loss of an essential 4.1 protein binding partner co-depleted in the reaction. To address this issue we added back-purified recombinant 80-kDa 4.1R to depleted extracts. Strikingly, spindle, centrosome, and aster reconstitution was completely restored by supplementation with recombinant 4.1R in extracts depleted with either SABD or C-terminal domain IgGs, producing structures with morphology and NuMA distribution comparable with controls (Fig. 3, A’, B’, and C’). The rescue of assembly by recombinant 4.1R shows directly that protein 4.1 is essential for the assembly of mitotic spindles, centrosome, and self-organized microtubule asters. Furthermore, this result indicates that a single isoform containing both SABD and C-terminal domains is sufficient to mediate all of the functions of 4.1 necessary for its role in organizing mitotic microtubule arrays in Xenopus egg extracts.
Protein 4.1 Domains in Spindle and Centrosome Assembly

Fig. 3. Aberrant spindle, centrosome aster, and self-organized aster assembly in vitro in 4.1-depleted extracts and rescue of defective phenotype by supplementation with recombinant 80-kDa 4.1. In the merged images, DNA in spindles was detected with 4,6-diamidino-2-phenylindole (blue), microtubules are red, and NuMA is green. Yellow indicates an overlap of red and green signals. A, the products of spindle assembly reactions in Xenopus extracts depleted using SABD IgG (ΔSABD) or C-terminal domain IgG (ΔC-terminal domain) were severely perturbed relative to spindles assembled in control extracts (IgG). Bar, 20 μm. B, centrosome asters assembled in Xenopus extracts depleted using SABD IgG or C-terminal domain IgG were aberrant both with respect to microtubules and NuMA distribution relative to centrosome asters assembled in control extracts. C, self-organized microtubule asters formed after Me2SO addition to extracts depleted using SABD IgG or C-terminal domain IgG were disoriented and had a scattered distribution of NuMA epitopes. A’, B’, C’, spindles, centrosome asters, and self-organized asters assembled after the addition of recombinant 80-kDa 4.1 (4.1R) to depleted extracts. With the addition of 9 μg of 80-kDa 4.1 spindles, centrosomes, and self-organized asters had similar morphology, size, and distribution of NuMA as to their respective controls.

4.1N 16–17

---KPRQTVKYPVR---QGVLEDWPVLVRKAGDIKLEKLKosexDKRERKYSK

4.1R 16–17

KKRLEQCNKIYHRNMLHLDQDGESREKEDKELSKFPK SGEEQDSKLICENSE

4.1R 16–17 NF

KKRLEQCNKIYHRNMLHLDQDGESREKEDKELSKFPK "NF"--SVEFEPSPSWDKLSTWT

C-terminal domain related peptides

4.1R 20–21

TVKGSIESTIKREIKRIVITQGADIDHEQVLQAIKEAEKEDQGDNVTVVVQRTFIA

4.1R 20–21 mut3V

TVKGSIESTIKREIKRIVITQGADIDHEQVLQAIKEAEKEDQGDNQFAVTQRTFIA

Fig. 4. Amino acid sequences of expressed peptides related to 4.1 SABD and C-terminal domain added to in vitro spindle, centrosome-nucleated, and self-organized aster assembly reactions. The 4.1R SABD peptide corresponds to a region encoded by exons 16–17. The 4.1N 16–17 peptide corresponds to the 4.1R SABD region but with low homology except for the boxed amino acids. The 4.1R 16–17NF has amino acids identical to 4.1R 16–17 (wild type 4.1R SABD) except for deletions of asparagine and phenylalanine in exon 17 (indicated by asterisks). The 4.1R C-terminal domain peptide corresponds to a region encoded by exons 20–21, and the 4.1R 20–21 mut3V peptide has an identical sequence except that three valines were mutated to alanines (underlined) This figure is modified from Krauss et al. (19).

Dominant Negative 4.1 Peptides Distort Assembly of Spindles, Centrosomes, and Microtubule Asters in Vitro—Having established that 4.1 is required for proper formation of microtubule structures, we next wanted to test the functions of specific 4.1 domains in spindle, centrosome, and microtubule aster assembly. To this end we added to in vitro assembly reactions bacterially expressed peptides with amino acid sequences corresponding to 4.1 domains. We reasoned that the peptides might act competitively to disrupt 4.1 complexes or to sequester important 4.1 binding partners during the assembly process. We analyzed peptide effects both on morphology and localization of the 4.1 binding partner NuMA as another measure of functional disruption because in controls NuMA localized in a tight focus at spindle poles, in the pericentriolar area of centrosomes and at the centers of self-organized asters (Fig. 3).

Initially we analyzed the effects of peptides related to 4.1 SABD and C-terminal domains. We focused on these domains because they (a) have important defined functions, (b) are highly conserved between frog and mammals, (c) were present in recombinant 4.1R used to rescue extracts immunodepleted by SABD and C-terminal domain IgGs, and (d) were demonstrated to profoundly distort nuclear assembly in vitro in egg extracts (19). For our experiments, we expressed His6-tagged peptides encoded by either exons 16–17 (amino acids 644–705) in the 4.1R spectrin-actin binding domain (SABD) or by exons 20–21 (amino acids 800–858) of the 4.1R C-terminal domain (Fig. 4). As controls for the SABD peptide, we used a variant 4.1N SABD peptide with low amino acid sequence homology to the 4.1R 16–17 peptide and a 4.1R 16–17ΔNF peptide with a deletion of two amino acids within its actin binding domain rendering it unable to bind actin but retaining spectrin binding. As a control for the 4.1R C-terminal domain peptide we used a C-terminal domain peptide in which three valines were changed to alanines (underlined) This figure is modified from Krauss et al. (19).

Addition of the 4.1R 16–17 SABD peptide produced spindles with less focused microtubules at the poles and more dispersed NuMA in the peripolar area. Often there were multipolar structures. In these spindles, chromatin appeared less condensed at the metaphase plate (Fig. 5A, top row). Centrosome asters, while retaining a small central focus of NuMA staining, had only a few bundled microtubules radiating outward (Fig. 5A, middle row). Self-organized asters also appeared to contain bundled microtubules and had a very diffuse central area of NuMA with additional NuMA epitopes distributed along microtubule bundles (Fig. 5A, bottom row). However, an equal
The concentration of a deletion mutant in the 4.1R SABD peptide (4.1R 16–17/H9004 NF) added to extracts did not affect the assembly of spindles, centrosomes, or asters with respect to the morphology or distribution of NuMA (Fig. 5A). A variant 4.1N SABD peptide also did not cause any apparent perturbation (Fig. 5A). Therefore the dominant negative effects of the 4.1R SABD peptide on proper structural assembly are sequence-specific and require its capacity to bind actin.

The C-terminal domain was also critical for the assembly of normal spindles, centrosomes, and microtubule asters. C-terminal domain peptide addition resulted in structures even more distorted than those treated with SABD peptides. Spindles had unfocused poles or were multipolar and were also bent in most cases. Centrosome-nucleated and self-organized asters were not radiating from a discernable center, but appeared as microtubule "mats" with irregularly distributed NuMA (Fig. 5A). Although the importance of NuMA in proper spindle formation is well documented, these observations imply that the interaction of NuMA and 4.1 is also crucial for centrosome and microtubule aster formation in vitro. To test this, the mutant C-terminal domain peptide with decreased NuMA affinity was added to the assembly reactions. Surprisingly this peptide had dramatic and unpredicted effects. In both centrosome and aster assembly reactions, no microtubules of normal length were observed but only small NuMA foci were detected with extremely short and sparse microtubules (Fig. 5A). In spindle reactions, Xenopus sperm DNA was decondensed and had juxtaposed a single polar remnant. Quantification of these effects shows that dominant negative effects of 4.1R SABD and C-terminal domain peptides on microtubule-based structures are profound and sequence-specific (Fig. 5B).

Protein 4.1 Function Is Required Continuously to Maintain Proper Microtubule Organization in Mitotic Structures—Our results indicate that 4.1 is essential for generating properly polymerized and organized microtubule asters and spindles. We wanted to test whether 4.1 function becomes dispensable once structures have assembled. This question could easily be addressed in Xenopus egg extracts, because inhibitory peptides
Fig. 6. Effects of 4.1 peptides on structural maintenance of mature spindles and self-organized microtubule asters. A, after assembly of spindles in extracts 4.1 peptides were added and incubated for an additional 15 min. Structures were spun onto coverslips and analyzed by immunofluorescence. In the merged images, DNA was stained by 4,6-diamidino-2-phenylindole (blue), microtubules are red, and NuMA is green. Bar, 20 μm. B, microtubule asters formed after an addition of 5% Me2SO to extracts were further incubated for 15 min with 4.1 peptides, and structures were spun onto coverslips. Coincidence of red (microtubules) and green (NuMA) signals generates a yellow coloration. Bar, 15 μm.

could be added at any time to assembly reactions.

We tested whether microtubules in bipolar spindles and self-organized asters could survive exposure to C-terminal domain mut3V peptides by adding C-terminal domain mut3V peptides to extracts containing normal preformed structures. After a 15-min incubation, few microtubules remained in spindles. These microtubules appeared to extend from pole remnants demarcated by very small NuMA-staining foci and were markedly “slackened.” Chromatin was decondensed and not positioned at a spindle equator, extending beyond the microtubules (Fig. 6A). Incubation with wild type C-terminal domain peptide also compromised structure but much less severely than C-terminal domain mut3V mutant peptides. Spindle microtubules became less rigidly and symmetrically organized with smaller less focused NuMA staining in the pale areas. Some NuMA was also unevenly distributed on chromatin (Fig. 6A). Incubation with SABD peptides dispersed peripolar NuMA, whereas SABDΔNF peptides did not appear to compromise spindle morphology or NuMA localization.

To test more stringently the effects of 4.1 C-terminal domain peptides on microtubules arrayed independently of centrosomes, self-organized asters stabilized by Me2SO were incubated with 4.1 peptides. After the exposure of microtubule asters to C-terminal domain mut3V peptides, there remained on average 3–5 microtubule bundles emanating from a NuMA-containing area similar in size to controls (Fig. 6B). Incubation with wild type C-terminal domain peptides produced a different structure phenotype having markedly truncated microtubules symmetrically arrayed around a NuMA region less tightly focused than in controls. In some orientations, a central hollow area or ring-like distribution of NuMA was detected (Fig. 6B). Because most mature microtubules in spindles as well as self-organized asters did not survive exposure to either mutant or wild type C-terminal domain peptides, it appears that continuous 4.1 function is required to maintain proper microtubule polymerization and organization.

The deleterious effects on mature spindles and asters could result from direct or indirect microtubule destabilization mechanisms. To investigate whether 4.1 peptides directly bind or destabilize microtubules, we performed a sedimentation assay using purified tubulin polymerized in vitro that compares the polymerized tubulin polymer (in the pellet) with unpolymerized tubulin (in the supernatant fraction). In control reactions without tubulin, none of the 4.1-related peptides pelleted under microtubule polymerizing conditions (Fig. 7, −tub lanes). When added to microtubule polymerizing reactions, SABD peptides remained entirely in the supernatant and did not appreciably alter the amount of microtubules pelleted. In reactions with either C-terminal domain or C-terminal domain mut3V peptides, each peptide was detected both in the microtubule pellet and in the supernatant (Fig. 7, +tub lanes). However, the amount of tubulin in the supernatant and pellet was similar to controls or when SABD peptide was added. Therefore, whereas both C-terminal domain-related peptides showed some binding to microtubules and SABD peptides did not, the 4.1 peptides did not have measurable direct microtubule destabilizing activity.

**DISCUSSION**

**Protein 4.1 Is Essential for Spindle and Centrosome Assembly**—Protein 4.1 interactions in mammalian red cells serve to link and stabilize structural components in the membrane skeleton with integral membrane proteins. Protein 4.1 is also widely distributed in many tissues and localizes at several subcellular sites crucial for cell division in nucleated cells. By analogy to its critical role in red cell membrane mechanics, protein 4.1 may link or stabilize components in nuclei, centrosomes, and mitotic spindles, providing both structural organization and flexibility necessary for dynamic cytoskeletal assembly and disassembly during the cell cycle. To begin to address 4.1 function in microtubule morphogenesis, we exploited the power of in vitro reconstitution using Xenopus egg extracts after first establishing 4.1 localization at in vitro assembled spindles, centrosomes, and self-organized microtubule asters. In depletion/add-back experiments, markedly aberrant structures formed in depleted egg extracts, but the addition of purified recombinant 80-kDa 4.1R protein restored normal centrosome and spindle assembly demonstrating that 4.1 is essential. Because 4.1 was required for arraying microtubule asters by motor-driven self-organization as well as by microtubule
nucleating centrosomes, the requirement for 4.1 to properly organize and orient microtubules is not dependent exclusively on centrioles. Although rescue was with recombinant 80-kDa 4.1R containing both a SABD and C-terminal domain, it is possible that 4.1 rescue may not be exclusive to that isoform or even to the 4.1R family. Multiple 4.1R splice variants exist, and recent transfection studies (16) showed that apparent co-localization of 4.1R with microtubules or disruption of microtubule organization may be isoform- and tissue-specific. Furthermore, another generally expressed 4.1 family member (4.1G) also contains an SABD region that can form a ternary complex with spectrin and actin (36) and a highly homologous 4.1G C-terminal domain that can bind NuMA (37). Because 4.1G colocalizes with NuMA at spindle poles (37) and has been detected at centrosomes,2 future experiments will test the rescue of spindle and centrosome assembly in 4.1-depleted extracts using specifically engineered 4.1 recombinant proteins from both the 4.1R and 4.1G families. This approach will facilitate functional mapping of 4.1 sequences required for microtubule organization in addition to the microtubule binding site in the 4.1R FERM (16).

4.1 Peptides From Two Independent Domains Act as Dominant Negatives in Assembly Reactions—To begin to define 4.1 domains critical for aster/spindle assembly and structure we added peptides to egg extracts, initiated assembly and examined morphology of the structures formed. One domain that behaved as a dominant negative was the SABD peptide. Although neither F-actin nor spectrin is reported to be a centrosome or spindle component, an SABD peptide mutant for actin binding did not exert deleterious effects on morphology. This suggests several of the following possibilities. 1) The SABD may be able to interact with centractin/Arp1, a protein >50% homologous to actin required for centrosomal microtubule anchoring and/or focusing (38, 39). Centractin/Arp1 is also a major component of dynactin, an activator of the minus end-directed microtubule motor cytoplasmic (40, 41). This is a particularly attractive hypothesis because disruption of dynein-dynactin complexes or overexpression of centractin/Arp1α perturbs centrosomes and spindles (25, 42, 43). 2) Interactions between microtubules and actin may contribute to aspects of spindle and centrosome formation. Proteins like MACF have been identified that cross-link these two filaments (44). 3) Non-filamentous actin, present in nuclei (45, 46) could play a role in spindle and centrosome assembly. It was reported that one molecule of actin is present for each dynein complex (47). Future experiments could test whether actin inhibitors such as latrunculin interfere with spindle and centrosome assembly. 4) The two deleted amino acids in SABD peptides, critical for actin binding (36), may also be part of another interaction site for an unidentified 4.1 binding partner.

The discovery of a NuMA binding site within exons 20–21 of the 4.1R C-terminal domain and that NuMA, dynein, and the dynactin subunit p150/glu co-precipitate with 4.1 (17) provided us with insights about possible mechanisms responsible for the dominant negative effects of C-terminal domain peptides on spindle and centrosome assembly. Transport of NuMA toward microtubule minus ends is required for assembly and maintenance of focused spindle poles (25); aster C-terminal domain peptides may disengage NuMA/dynein as cargo from the dynein motor. This is not unlikely, because the transient nature of the interaction of dynein with dynactin/NuMA has been reported (25, 40). Additionally, NuMA also directly binds to and stabilizes microtubules (48), but stabilization is regulated by other proteins such as LGN (49). Although the neighboring 4.1 binding site on NuMA does not directly overlap the microtubule/LGN binding site of NuMA, 4.1 C-terminal domain peptides may indirectly alter the modulation of microtubule dynamics integral to the assembly of spindle poles and asters. The dense microtubule mats with mislocalized NuMA observed after centrosome or aster assembly with C-terminal domain peptides may reflect NuMA oligomerization and stabilization of microtubules (48, 49).

Protein 4.1 and the Minus Ends of Microtubules—Localization of 4.1 at the minus ends of radially arrayed microtubules in spindles and centrosomes may occur by two mechanisms: 1) transport by minus end-directed motors and/or 2) association with other minus end-associated proteins already on site (reviewed in Ref. 50). At spindle poles/centrosomes 4.1 could function not only as a crucial structural protein but also to regulate interactions within scaffolding complexes such as those containing NuMA (17), γ-tubulin (15), and pericentrin (1). Some of these interactions appear to be dynamic, because as reported here 4.1 function is required for continuous maintenance of proper mitotic structures. However, many key functions of 4.1 related to microtubules remain to be identified as suggested by another recent study (37) as well as demonstrated by our own data.

In mitotic egg extract assembly reactions, few microtubules survived exposure to C-terminal domain peptides containing changes in only three amino acids critical for NuMA binding. When added to nuclear assembly reactions in interphase egg extracts, this peptide also produced markedly truncated centrosome/microtubule arrays (19). However, C-terminal domain mut3V peptides did not directly destabilize microtubules polymerized from purified tubulin and, in fact, partially co-pelleted with them. This observation indicates the involvement of other extract proteins in co-regulation of microtubule dynamics by 4.1.

Our investigations predict that multiple protein 4.1 domains regulate microtubule dynamics and/or release as well as microtubule organization. Apparently this is accomplished by both direct and indirect mechanisms. Supporting the hypothesis that 4.1 impacts cell division, depletion of 4.1 or addition of 4.1 dominant negative peptides produced multipolar asymmetric spindles and strongly disrupted centrosome organization. Clearly 4.1 misregulation or disruption in vivo could lead to spindle and centrosomal aberrations, abnormal chromosome segregation, and cell cycle perturbations, hallmarks of potential pathology.

Acknowledgments—We thank Drs. A. Merdes, T. Schroer, M. Welch, and X. An for valuable discussions. We also thank members of the Heald laboratory, especially Dr. S. Wignall, for helpful suggestions. We thank R. Couto for final preparation of the figures.

REFERENCES

1. Krauss, S. W., Chasis, J. A., Rogers, C., Mohandas, N., Krouchmalnic, G., and Pennman, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7297–7302
2. Krauss, S. W., Larabell, C. A., Lockett, S., Gascard, P., Mohandas, N., and Chasis, J. A. (1997) J. Cell Biol. 137, 275–289
3. DeCarre, G., Lallena, M. J., and Carreus, I. (1985) Biochem. J. 312, 871–877
4. Conboy, J. G., Chan, J., Mohandas, N., and Kan, Y. W. (1988) Proc. Natl. Acad. Sci., U. S. A. 85, 9062–9065
5. Chasis, J. A., Coulombe, L., Conboy, J., McGee, S., Andrews, K., Kan, Y., and Mohandas, N. (1993) J. Clin. Investig. 91, 329–338
6. Horne, W. C., Prinz, W. C., and Tang, E. K. (1990) Biochem. Biophys. Acta 1055, 87–92
7. Huang, J. P., Tang, C. J., Kog, G. H., Marchesi, V. T., Benz, E. J., and Tang, T. K. (1993) J. Biol. Chem. 268, 3758–3766
8. Subrahmanyan, G., Bertos, P. J., and Anderson, R. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9222–9226
9. Walensky, L. D., Gascard, P., Fields, M. E., Blackshaw, S., Conboy, J. G., Mohandas, N., and Snyder, S. H. (1998) J. Cell Biol. 141, 143–153
10. Walensky, L. D., Blackshaw, S., Liao, D., Watkins, C. C., Weier, H. U., Parra, M., Huganir, R. L., Conboy, J. G., Mohandas, N., and Snyder, S. H. (1999) J. Neurosci. 19, 6457–6467
11. Parra, M., Walensky, L., Chan, N., Snyder, S., Mohandas, N., and Conboy, J. (1998) Cell 93, 265a
12. Parra, M., Gascard, P., Walensky, L. D., Snyder, S. H., Mohandas, N., and Conboy, J. G. (1998) Genomics 49, 298–306
13. Parra, M., Gascard, P., Walensky, L. D., Gimm, J. A., Blackshaw, S., Chan, N.,

2 S. W. Krauss, unpublished data.
Protein 4.1 Domains in Spindle and Centrosome Assembly

14. Gascard, P., Lee, G., Coulombel, L., Affray, I., Lum, M., Parra, M., Conboy, J. G., Mohandas, N., and Chasis, J. A. (1998) Blood 92, 4404–4414
15. Hung, L. Y., Tang, C. J., and Tang, T. K. (2000) Mol. Cell. Biol. 20, 7813–7825
16. Perez-Ferreiro, C. M., Luque, C. M., and Correas, I. (2001) J. Biol. Chem. 276, 44785–44791
17. Mattagajasingh, S. N., Huang, S. C., Hartenstein, J. S., Snyder, M., Marchesi, V. T., and Benz, E. J., Jr. (1999) J. Cell Biol. 145, 29–43
18. Krauss, S. W., Chen, C., Pennman, S., and Heald, R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 10752–10757
19. Krauss, S. W., Heald, R., Lee, G., Nunomura, W., Gimm, J. A., Mohandas, N., and Conboy, J. G., Mohandas, N., and Chasis, J. A. (1998) Blood 92, 4404–4414
20. Kiseleva, E., Drummond, S. P., Goldberg, M. W., Rutherford, S. A., Allen, T. D., and Wilson, K. L. (2004) J. Cell Sci. in press
21. Merdes, A., and Cleveland, D. W. (1997) J. Cell Biol. 138, 953–956
22. Compton, D. A., Szilak, I., and Cleveland, D. W. (1992) J. Cell Biol. 116, 1395–1408
23. Yang, C. H., Lambie, E. J., and Snyder, M. (1992) J. Cell Biol. 116, 1303–1317
24. Gaglio, T., Saredi, A., and Compton, D. A. (1995) J. Cell Biol. 131, 693–708
25. Merdes, A., Heald, R., Samejima, K., Earnshaw, W. C., and Cleveland, D. W. (2000) J. Cell Biol. 149, 851–862
26. Merdes, A., Ramyar, K., Vechio, J. D., and Cleveland, D. W. (1996) Cell 87, 447–458
27. Gaglio, T., Saredi, A., Bingham, J. B., Hashani, M. J., Gill, S. R., Schroer, T. A., and Compton, D. A. (1996) J. Cell Biol. 135, 399–414
28. Ashford, A., Andersen, S., and Hyman, A. (1998) in Preparation of Tubulin from Bovine Brain. A Laboratory Handbook (Biology, I. C., ed) Academic Press, San Diego
29. Murray, A. (1991) Methods Cell Biol. 36, 581–605
30. Sawin, K. E., and Mitchison, T. J. (1991) J. Cell Biol. 112, 941–954
31. Sawin, K. E., and Mitchison, T. J. (1994) Mol. Biol. Cell 5, 217–226
32. Moudjou, M., and Bornens, M. (1998) Method of Centrosome Isolation from Cultured Animal Cells, Cell Biology: A Laboratory Handbook
33. Heald, R., Tournebize, R., Habermann, A., Karsenti, E., and Hyman, A. (1997) J. Cell Biol. 138, 615–628
34. Wignall, S., Gray, N., Chang, Y.-T., Juarez, L., Jacob, R., Burlingame, A., Schultz, P., and Heald, R. (2004) Chem. Biol. 11, 135–146
35. Winardi, R., Discher, D., Kelley, C., Zen, L., Mays, K., Mohandas, N., and Conboy, J. G. (1995) Blood 86, 4315–4322
36. Gimm, J. A., An, X., Nunomura, W., and Mohandas, N. (2002) Biochemistry 41, 7275–7282
37. Delhommeau, F., Vasseur-Godbillon, C., Leclerc, P., Schischmanoff, P. O., Croisille, L., Rince, P., Moriniere, M., Benz, E. J., Jr., Tchernia, G., Tama-gnini, G., Ribeiro, L., Delaunay, J., and Baklouti, F. (2002) Blood 100, 2629–2636
38. Quintyne, N. J., and Schroer, T. A. (2002) J Cell Biol. 159, 245–254
39. Clark, S. W., and Meyer, D. I. (1992) Nature 359, 246–250
40. Schroer, T. A., and Sheetz, M. P. (1991) J. Cell Biol. 115, 1309–1318
41. Gill, S. R., Schroer, T. A., Szilak, I., Steuer, E. R., Sheetz, M. P., and Cleveland, D. W. (1991) J. Cell Biol. 115, 1639–1650
42. Quintyne, N. J., Gill, S. R., Eckley, D. M., Crevo, C. L., Compton, D. A., and Schroer, T. A. (1999) J. Cell Biol. 147, 321–334
43. Clark, I. B., and Meyer, D. I. (1999) J. Cell Sci. 112, 3507–3518
44. Sun, D., Leung, C. L., and Liem, R. K. (2001) J. Cell Sci. 114, 161–172
45. Rando, O. J., Zhao, K., and Crabtree, G. R. (2000) Trends Cell Biol. 19, 92–97
46. Pederson, T., and Aebi, U. (2002) J. Struct. Biol. 140, 9–9
47. Schafer, D. A., Gill, S. R., Cooper, J. A., Heuser, J. E., and Schroer, T. A. (1994) J Cell Biol. 126, 403–412
48. Haren, L., and Merdes, A. (2002) J. Cell Sci. 115, 1815–1824
49. Du, Q., Taylor, L., Compton, D. A., and Macara, I. G. (2002) Curr. Biol. 12, 1928–1933
50. Dammermann, A., Desai, A., and Oegema, K. (2003) Curr. Biol. 13, 614–624
AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES

A—If this running head is not acceptable, please provide one of 60 characters or less, including spaces.

B—The Journal guidelines state that the summary should fit into the left-hand column. If your summary is long enough to run over into the right-hand column, please cut text.

C—Does this need a reference citation for previous work?

D—If ‘present in’ has changed the scientific meaning, please note.

E—Per Journal style most abbreviations, such as ‘CSF’ here, in the summary and text must be used a minimum of three and five times, respectively, or written out without the abbreviation.

F—Journal uses ‘Me₂SO’ instead of ‘DMSO’ and no definition is necessary, although it can be added to the abbreviations footnote.

G—Please confirm complete affiliation here for EMBL.

H—Changed ‘μm’ to ‘μM’ tubulin. Confirm or correct.

I—If ‘isoform-specific’ is not meant here, please delete hyphen.

J—Per Journal style, unpublished data citations are moved to a footnote. Please confirm/correct author name and add additional authors if necessary.

K—The numeration has been adjusted per Journal style to accommodate multiple sentences in the series. Please confirm that the numbering associated with the points outlined is correct.

L—Journal avoids possessive tense.

M—Journal does not use courtesy titles such as ‘Mr.’ and ‘Mrs.’.

N—Ref. 20: Can you supply volume and page numbers now?

O—Ref. 28: Please provide a page range for the portion of the book used.

P—Ref. 32 is missing information. Please supply page range if this refers to a portion of the handbook. If that is the entire name of the handbook, please confirm. Please supple editors’ names if applicable. Please supply publisher and city of publication.

Q—For Figs. 1-3, 5, and 6, please check colors on the computer screen against what appears in the
legend and amend the legend as necessary. Are the color figures acceptable for publication? If not, please send a good quality high resolution hard copy to be scanned. We cannot accept digital art at this stage.

R—If ‘detected’ is not a suitable verb here, please provide another.