Asymmetric mitosis: Unequal segregation of proteins destined for degradation

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Mitotic cell division ensures that two daughter somatic cells inherit identical genetic material. Previous work has shown that signaling by the Smad1 transcription factor is terminated by polyubiquitinylation and proteasomal degradation after essential phosphorylations by MAPK and glycogen synthase kinase 3 (GSK3). Here, we show that, unexpectedly, proteins specifically targeted for proteasomal degradation are inherited preferentially by one mitotic daughter during somatic cell division. Experiments with dividing human embryonic stem cells and other mammalian cultured cell lines demonstrated that in many supposedly equal mitoses the segregation of proteins destined for degradation (Smad1 phosphorylated by MAPK and GSK3, phospho-β-catenin, and total polyubiquitinylated proteins) was asymmetric. Transport of pSmad1 targeted for degradation to the centrosome required functional microtubules. *In vivo*, an antibody specific for Mad phosphorylated by MAPK showed that this antigen was associated preferentially with one of the two centrosomes in *Drosophila* embryos at cellular blastoderm stage. We propose that this remarkable cellular property may be explained by the asymmetric inheritance of peripheral centrosomal proteins when centrioles separate and migrate to opposite poles of the cell, so that one mitotic daughter remains pristine. We conclude that many mitotic divisions are unequal, unlike what was previously thought.

centrosome | polyubiquitin | proteasome | Smad | β-catenin

Since the description of mitosis by Flemming in 1882, studies on somatic cell division have focused on the equal partition of cellular materials, in particular that of the chromosomes and mitotic apparatus, between cell daughters (1, 2). We now report that many mitotic divisions are unequal with respect to pericentrosomal proteins targeted for proteasomal degradation. The starting point for this investigation was provided by recent work showing that the duration of the Smad1 signal triggered by bone morphogenetic proteins (BMPs) is controlled by an elaborate protein degradation pathway (Fig. 1*A*). After activation via C-terminal phosphorylation by BMP receptor (BMPR), Smad1 is subjected to sequential phosphorylations by MAPK and glycogen synthase kinase 3 (GSK3) in its linker (middle) region. The MAPK and GSK3 phosphorylations are both required for the polyubiquitinylation and degradation of Smad1 (Fig. 1*A*) (3, 4). We developed potent phospho-specific antibodies for positions Ser-214 (pSmad1^{MAPK}) and Ser-210 (pSmad1^{GSK3}) that allow one to follow the intracellular location of Smad1 protein that has been specifically targeted for degradation (4).

As is well known, centrosomes ensure that cells divide equally at mitosis and contain small centriole doublets surrounded by a matrix of proteins such as γ-tubulin and pericentrin that serve as the microtubule-organizing center (MTOC) (2). In recent years, the realization has emerged that the centrosome also functions as the proteolytic center of the cell. The key to this discovery was the finding that cultured mammalian cells treated with proteasome inhibitors display a massive increase of peripheral centrosomal material caused by the accumulation of undegraded proteins (5, 6). Proteasomes are normally concentrated in the centrosome of many cultured cell lines (7). In addition, it has been recently reported that Smad1 marked for degradation becomes localized to the centrosomal region of Cos7 cells (4).

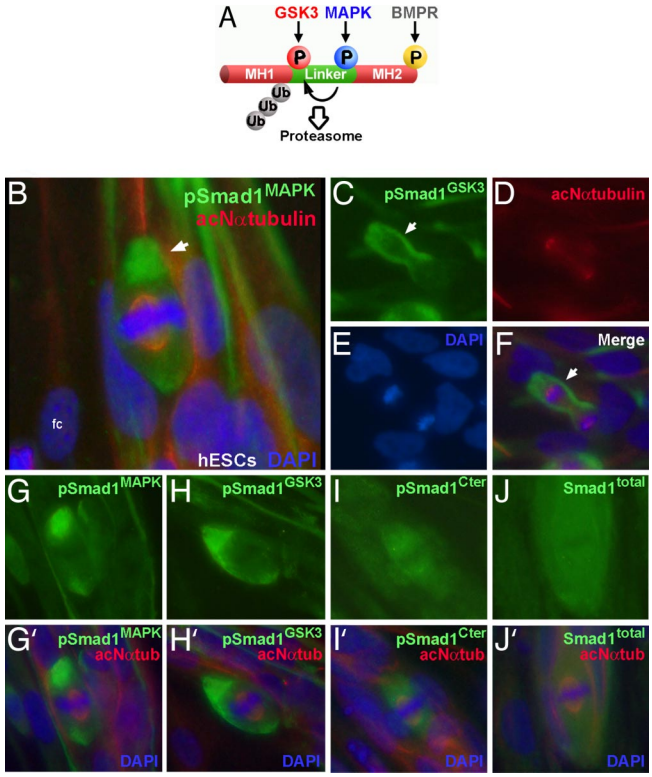

Fig. 1. Phospho-Smad1 forms targeted for degradation are asymmetrically segregated in dividing hESCs. (*A*) Diagram summarizing previous work showing that the Smad1 transcription factor is regulated by three successive phosphorylations followed by polyubiquitinylation and destruction in the proteasome (3, 4). (*B*) Asymmetric distribution of pSmad1^{MAPK} during metaphase. *N*-acetylated-α-tubulin marks the mitotic spindle. (*C–F*) Asymmetric pSmad1^{GSK3} distribution during anaphase. Arrows indicate the side containing more pSmad antigen; feeder cells (fc) were negative, facilitating the hESC analyses. (*G–J′*) Note that at metaphase pSmad1^{MAPK} and pSmad1^{GSK3} were asymmetric, pSmad1^{Cter} was only slightly asymmetric, and total Smad1 antigen was uniform. (Magnifications: *B*, ×520; *C–J*, ×320.)

In the present study, we show that, unexpectedly, proteins targeted for degradation are inherited preferentially by one

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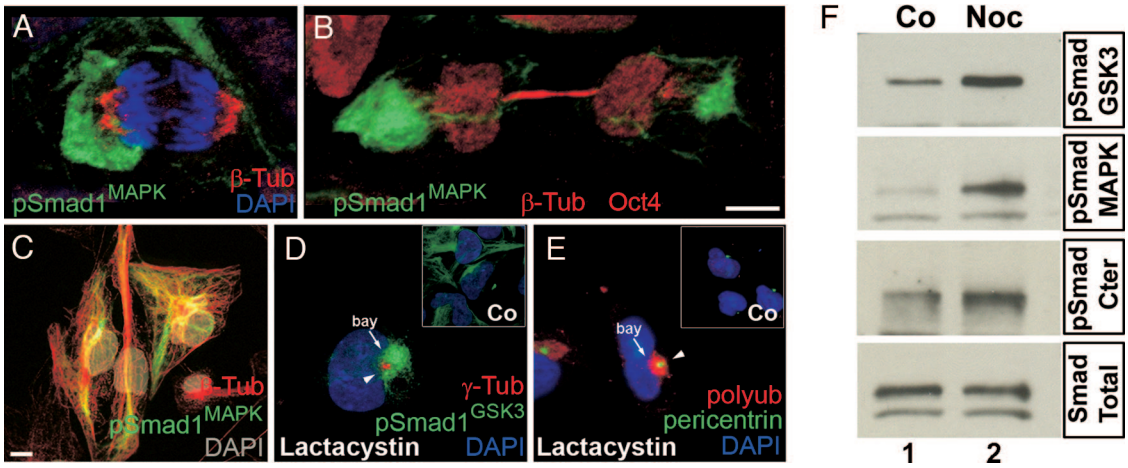

Fig. 2. hESCs mitoses with asymmetric pSmad1 marked for degradation are self-renewing divisions, and pSmad1 degradation in centrosomes requires functional microtubules. (*A*) At telophase, pSmad1^{MAPK} is asymmetric but not associated with the mitotic apparatus. (*B*) Both nuclei of asymmetric hESC daughters are Oct4-positive stem cells; β-tubulin marks the midbody. (*C*) pSmad1^{MAPK} antigen (in green) colocalizes with microtubules (in red) in hESCs. The DNA stain DAPI is shown in gray. (*D*) pSmad1^{GSK3} antigen concentrates greatly in the nuclear bay region (arrow) that contains the centrosome stained with γ-tubulin (arrowhead) in lactacystin-treated hESCs. (*E*) Total polyubiquitinated proteins accumulate in the nuclear bay region (arrow) induced by inhibition of proteasomal activity by lactacystin, which contains the centrosome marked by pericentrin (arrowhead). (*Insets*) hESCs were not treated with the proteasomal inhibitor. CO, control. (*F*) Western blot analysis of hESCs treated with nocodazole; note that phosphorylated forms of Smad1 increase with microtubule depolymerization, whereas total Smad1 remains unchanged. (Scale bar: 10 μm.)

daughter during cell division. When dividing human embryonic stem cells (hESCs) were immunostained, it was found that in 80–90% of mitoses pSmad1^{MAPK} and pSmad1^{GSK3} were segregated asymmetrically, whereas total Smad1 was uniform. This asymmetry was surprising, because self-renewing divisions of hESCs were expected to be symmetrical (8). Using Cos7 cells we also showed that pSmad1^{MAPK} and pSmad1^{GSK3}, β-catenin phosphorylated by GSK3, and total polyubiquitinylated proteins were asymmetrically inherited. These asymmetric distributions were transmitted through at least three consecutive cell generations. Asymmetries in proteins destined for degradation can also occur *in vivo*, because *Drosophila* embryos at the blastoderm stage showed an asymmetric spot of Mad phosphorylated by MAPK near one of the two centrosomes. We propose a mechanistic model in which this remarkable cellular property is explained by the asymmetric inheritance of peripheral centrosomal proteins at the time the centrioles separate and migrate to opposite poles of the cell at the G_2/M transition. We conclude that many mitoses, previously thought to be equal, are unequal when examined with antibodies specific for proteins marked for degradation.

Results

Asymmetries in Self-Renewing hESC Divisions. We examined several mammalian cultured cell lines with antibodies specific for phospho-Smad1 targeted for degradation (4) and observed that hESCs stained the brightest. Unexpectedly, we noticed that pSmad1 targeted for degradation was asymmetrically distributed during mitosis in hESCs. The great majority of mitotic hESCs displayed asymmetric distribution of pSmad1^{MAPK} or pSmad1^{GSK3} antigens during metaphase (Fig. 1*B*) and anaphase (Fig. 1 *C*–*F*). One side of the dividing cell displayed strong diffuse cytoplasmic staining [see arrows in Fig. 1 *B*, *C*, and *F* and supporting information (SI) Fig. S1]. The frequency of mitotic asymmetry in hESCs was very high for both the pSmad1^{MAPK} (91% in metaphase, $n = 125$, 86% in anaphase, $n = 118$) and pSmad1^{GSK3} (82% in metaphase, $n = 110$, 77% in anaphase, $n = 104$) antigens. Remarkably, these asymmetries were specific for Smad1 marked for degradation, because C-terminal phosphorylated Smad1 (pSmad1^{Cter}, which is mediated by activated BMPR) was less asymmetric, and total Smad1, which has a large unphosphorylated reservoir (9), was entirely uniform (Fig. 1 *G*–*J*′). We conclude that the asymmetric segregation was specific for the subset of Smad1 molecules marked for polyubiquitinylation and degradation.

We next asked whether these asymmetric hESC mitoses represented self-renewing, equal divisions. Isolated hESCs are able to divide and survive when plated on feeder mouse fibroblasts in the presence of the Rho-kinase (ROCK) inhibitor Y-27632 (10). The resulting daughter cells were asymmetric with respect to pSmad1^{MAPK} and pSmad1^{GSK3} antigens (Fig. 2 *A* and *B*), yet both represented pluripotent stem cells (8), as shown by the equivalent staining of daughter nuclei with the stem cell marker Oct4 in Fig. 2*B*. Thus, the asymmetric segregation of Smad1 destined for degradation we observed took place during self-renewing divisions of stem cells, which were previously expected to be equal.

Smad1 Degradation Requires Microtubular Transport. In interphase hESCs, the pSmad1^{MAPK} and pSmad1^{GSK3} antigens were associated with long cytoskeletal filaments that costained with β-tubulin, representing microtubules that converge on the centrosome (Fig. 2*C*). In hESCs treated with lactacystin, an inhibitor of proteasomal enzymatic activity, Smad1 marked for degradation, as well as total polyubiquitinylated proteins, strongly accumulated in the periphery of the centrosomal MTOC marked by γ-tubulin and pericentrin (Fig. 2 *D* and *E*). We also noted that when proteasomal degradation is inhibited the cell nucleus adopted a kidney-like shape. The undegraded proteins occupied the cytoplasm adjoining the "nuclear bay" concavity (Fig. 2 *D* and *E*). It is classically known that the cytoplasm within nuclear bays always contains the centrosome (1). hESC cultures in which microtubules were depolymerized for 2 h with nocodazole showed increased levels of pSmad1^{Cter}, pSmad1^{MAPK}, and pSmad1^{GSK3}, whereas total Smad1 levels remained unaffected (Fig. 2*F*). In nocodazole-treated cells the phospho-Smad1 antigens lost their microtubular association and stained intensely the cytoplasm (data not shown). This distribution was very different from that of lactacystin-treated hESCs, in which the antigen concentrated in the periphery of the centrosome (Fig. 2*D*). We

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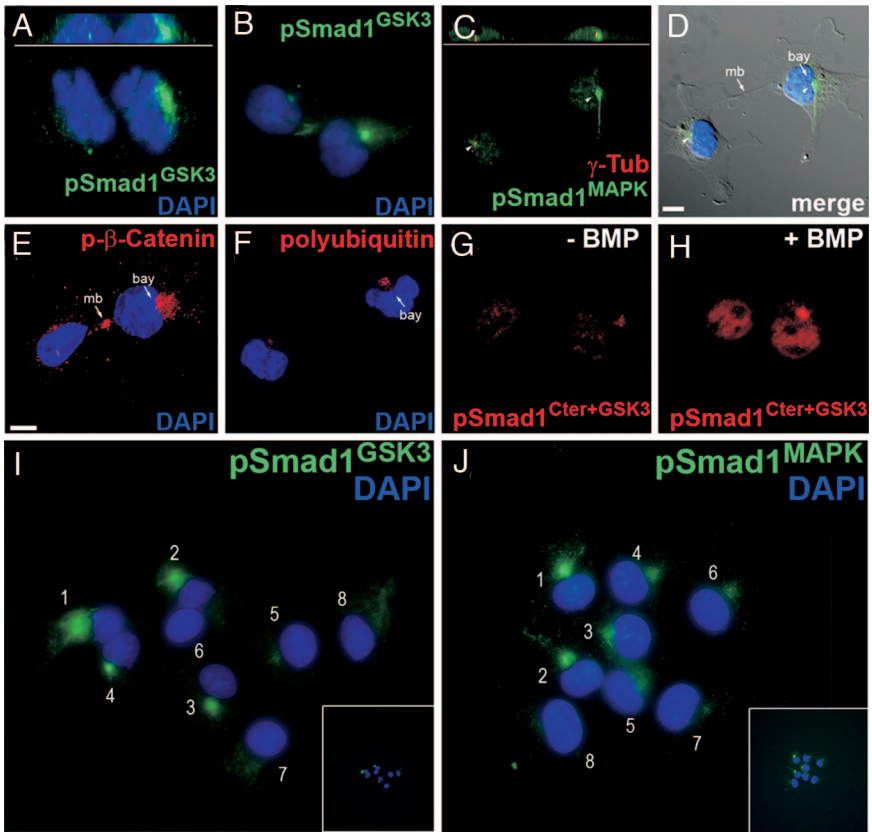

Fig. 3. Asymmetric distribution of pSmad1^{GSK3}, pSmad1^{MAPK}, p-β-catenin, and total polyubiquitinylated proteins in synchronized Cos7 daughter cells. (*A* and *B*) Asymmetric pSmad1^{GSK3} at early daughter cell stage. (*C* and *D*) Asymmetric pSmad1^{MAPK} after cell division. Note that γ-tubulin (red dots) is equally partitioned (arrowheads), whereas pSmad1^{MAPK} accumulates asymmetrically in the nuclear bay cytoplasm of one cell (arrow); these daughter cells are still joined by the midbody (mb). The z-stacks (shown as *Insets* at the top of *A* and *C*) show that the merged images include the entire cell. (*E* and *F*) β-Catenin phosphorylated by GSK3 and total polyubiquitinylated proteins are asymmetrically inherited and accumulate in nuclear bays. Note that the nuclear bays are also asymmetrical. (*G* and *H*) Cos7 daughter cells with asymmetric pericentrosomal pSmad1^{GSK3} respond to BMP7 treatment (5 nM for 90 min) by translocating equal levels of pSmad^{Cter} into the nucleus (see Fig. S3 for experimental details). (*I* and *J*) Clones of eight Cos7 cells cultured for 48 h after synchronization reveal successive asymmetric divisions of pSmad1^{GSK3} and pSmad1^{MAPK} through three consecutive cell cycles. (*Insets*) Low-power images demonstrating that asymmetric cells indeed derive from isolated clones. (Scale bar: 10 μm.)

conclude from these results that the degradation of pSmad1 normally requires microtubular transport to the centrosome.

Cos7 Daughter Cell Pairs Show Asymmetries. To investigate whether asymmetrical cell division also occurred in other mammalian somatic cell lines, we analyzed Cos7 cells, in which the pericentrosomal localization of pSmad1^{MAPK} and pSmad1^{GSK3} is prominent at interphase, particularly in nonconfluent G_1 phase cells (4). Cos7 cells were synchronized by mitotic shake-off (11) and plated at low density on Con A-coated slides. Shortly after synchronization, most cells were single and undergoing mitosis. As they divided, strong staining was seen on one side (Fig. 3*A*). Two to 4 h after mitotic synchronization, 71% of the cells formed daughter cell pairs (n = 850), sometimes still joined by microtubular midbodies (Fig. 3*D*). Thus, the shake-off synchronization method allows one to study the asymmetric distribution of proteins in cell daughters.

When pSmad1^{GSK3} and pSmad1^{MAPK} regained their normal centrosomal location during G_1, asymmetric distributions were observed for both antigens (Fig. 3 *B–D*). Double stainings with the MTOC marker γ-tubulin showed that Smad1 destined for degradation accumulated in the peripheral centrosomal material, whereas γ-tubulin was equally inherited (Fig. 3*C*). We also noted that this material frequently occupied the cytoplasm adjoining the nuclear bay that was present in only one daughter (Fig. 3*D*). The asymmetry in nuclear bay inheritance between cell daughters provides a useful cytological support for the asymmetry in cell divisions reported here. In the case of pSmad1^{GSK3} staining, Cos7 divisions were asymmetric in 44 $\pm$ 7% ($\pm$ SD) of cell pairs, 14 $\pm$ 5% were symmetric, and the rest stained below detectable levels and therefore their asymmetries could not be assessed (n = 220, four independent experiments). For pSmad1^{MAPK}, 56% of daughter cell pairs were asymmetric (n = 55). When only daughter cell pairs that stained above detection levels are considered, the high incidence of asymmetric cell pairs suggests that the process is not random in Cos7 cells. The case of hESCs described above, in which the incidence of asymmetries was $\approx$90% of all mitotic cells, also shows that the partition is nonrandom. As will be seen below, asymmetrical distribution not accompanied by a high incidence of equal distribution was also seen in *Drosophila* blastoderm embryos.

Phospho-β-Catenin and Total Polyubiquitinylated Proteins Segregate Asymmetrically. We next investigated whether this asymmetric inheritance was a more general phenomenon by analyzing other proteins targeted for degradation. β-Catenin is a regulatory protein that is polyubiquitinylated and degraded when phosphorylated by GSK3 (12, 13). Phospho-β-catenin accumulates in the periphery of the basal body of the primary cilium, which corresponds to the mother centriole (14, 15), providing an excellent candidate to test the inheritance of proteins in train of proteasomal degradation during mitosis. Immunostainings with

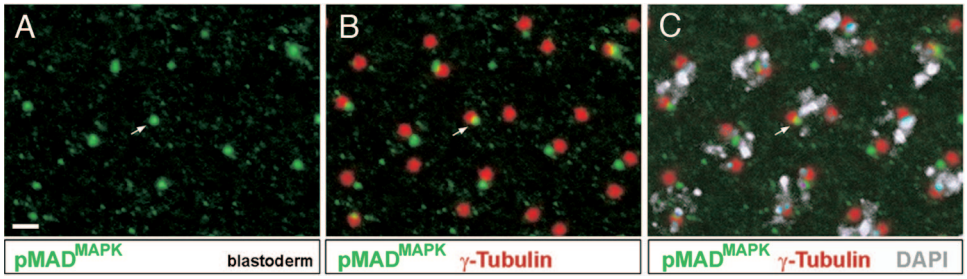

Fig. 4. pMad^{MAPK} is distributed asymmetrically *in vivo* in *Drosophila*. A rabbit polyclonal antibody reagent specific for *Drosophila* Mad phosphorylated by MAPK at its unique PXSP site was developed (see *Materials and Methods*). (*A*) High power view of *Drosophila* embryos at the cellular blastoderm stage stained with pMad^{MAPK} antibody. (*B*) Same field co-stained for γ-tubulin (red) and pMad^{MAPK}(green). (*C*) Triple staining including the DNA stain DAPI (gray). Note that each *Drosophila* blastoderm cell contains a single asymmetrical spot of accumulation of pMad^{MAPK} antigen adjacent or nearby one of the two centrosomes marked by γ-tubulin (we note that in few cells the intensity is lower). For low-power views see Fig. S4. (Scale bar: 2 μm.)

a monoclonal anti-β-catenin phospho-specific antibody directed against phospho-serines 33 and 37 (Sigma) revealed that phospho-β-catenin was asymmetrically inherited by daughter Cos7 cells (Fig. 3*E* and Fig. S2). This result also suggests that degradation by the β-catenin destruction complex (13) normally takes place in the pericentrosomal region. Asymmetric localization of phospho-β-catenin in the pericentrosomal region was observed in 52 ± 2% of Cos7 daughter cell pairs (n = 239, two independent experiments).

In addition to phospho-β-catenin, a mAb specific for polyubiquitin chains (16) revealed that total polyubiquitinylated proteins were asymmetrically inherited, localizing to the daughter cell with the largest nuclear bay (Fig. 3*F*). The polyubiquitin stainings imply that this degradation pathway is used by many other cellular proteins in addition to β-catenin and Smad1.

Strikingly, when clones of eight Cos7 daughter cells (obtained 48 h after mitotic synchronization) were stained for pSmad1 marked for degradation by GSK3 or MAPK phosphorylation, one cell was found to contain stronger centrosomal staining than the other seven, with a gradation between them (Fig. 3 *I* and *J*; the strongest stained cell is indicated as 1 and the weakest one as 8). Therefore, these asymmetries were propagated (with some imperfections) through three successive cell divisions in these clones. We note that we also observed several clones of seven cells, in which one was strongly stained; in these clones the accumulation of undegraded proteins in one cell may inhibit cell division and perhaps lead to apoptosis.

We conclude that cultured mammalian somatic cells have the unexpected ability of segregating pSmad1 marked for degradation, β-catenin phosphorylated by GSK3, and total polyubiquitinylated proteins preferentially into one of their daughters at cell division.

Mitotic Asymmetry Does Not Affect the Response to BMP Signals. We next tested the consequence of Smad1 asymmetries on BMP signaling in the resulting daughter cells. Using nuclear pSmad1^{Cter} antigen levels as a measure of BMP signaling (and centrosomal pSmad1^{GSK3} to mark the cell inheriting the Smad1 targeted for degradation), we determined that both Cos7 daughter cells responded equally to BMP7 treatment (5 nM for 90 min) by translocating the same levels of pSmad1^{Cter} into the nucleus (Fig. 3 *G* and *H* and Fig. S3). When cell pairs were counted, among the BMP-treated daughter cell pairs (n = 353) 41% had unequal centrosomal pSmad1^{GSK3} staining (whereas in 53% no centrosomal staining was detected and in 5% uniform staining was seen). The nuclear pSmad1^{Cter} signal was symmetric in 97% of cell pairs displaying unequal centrosomes (n = 146). These results indicate that the centrosomal pSmad1 does not bias the cellular response to a BMP7 stimulus (both daughter cells respond equally), as one might expect if this were a mechanism for segregating proteins in train of destruction to one daughter cell.

Phospho-Mad^{MAPK} Asymmetries in *Drosophila* Blastoderm. Because the asymmetric distributions were observed in cultured mammalian cell lines (hESC and Cos7 as well as L cells, data not shown) it was important to determine whether this phenomenon also occurred *in vivo*. To observe asymmetric localizations, we developed an antibody specific for *Drosophila* Mad (the Smad1/5/8 homologue) protein phosphorylated by MAPK. Mad contains a single canonical MAPK site (PXSP) at Ser-212 (4). This rabbit polyclonal antiserum (nonaffinity purified; see *Materials and Methods*) was entirely specific for Mad phosphorylated at this site because a Ser-to-Ala mutation eliminated reactivity (Fig. S4). The pMad^{MAPK} antigen was inhibited by phosphatase treatment and tracked Dpp signaling in a dorsal stripe of the embryo (Fig. S4). This antibody reagent revealed that in *Drosophila* embryos at the cellular blastoderm stage a single spot of pMad^{MAPK} was observed adjacent or nearby (but not surrounding) one of the two centrosomes marked by γ-tubulin in each cell (Fig. 4). At low-power views, it was evident that the overall number of centrosomes (n = 842) was double that of pMad^{MAPK} spots (n = 445), as shown in Fig. S5. At mitosis the pMad^{MAPK} antigen diffuses throughout the cell (data not shown), explaining why most cells contain a spot of pMad^{MAPK} in Fig. 4*A* (however, we note that a few weakly stained blastoderm cells were also observed, but at low frequency; these merit further investigation). These results in *Drosophila* embryos demonstrate that the asymmetric localization of protein destined for degradation near centrosomes can also occur *in vivo*.

Discussion

A Model for Mitotic Asymmetry. The results presented here were made possible by the development of high-titer, affinity-purified antibody reagents for Smad1 targeted for degradation by MAPK and GSK3 phosphorylations (4). These reagents revealed that a large proportion of somatic cell mitoses are asymmetrical, rather than entirely symmetrical as previously thought. An attractive working hypothesis, depicted in the cell biological model shown in Fig. 5, is that when the centrioles separate at the G_2/M phase to occupy opposite cell poles the peripheral centrosomal material remains on one side. We propose that the unequal distribution arises because this peripheral centrosomal material is inherited preferentially by one of the daughter cells (Fig. 5). In some cell types, the pericentrosomal material can be as large as the cell nucleus itself, as was recognized by early cytologists, who gave this material names such as "centrospheres" or "idiozomes" (see figure 11 in ref. 1).

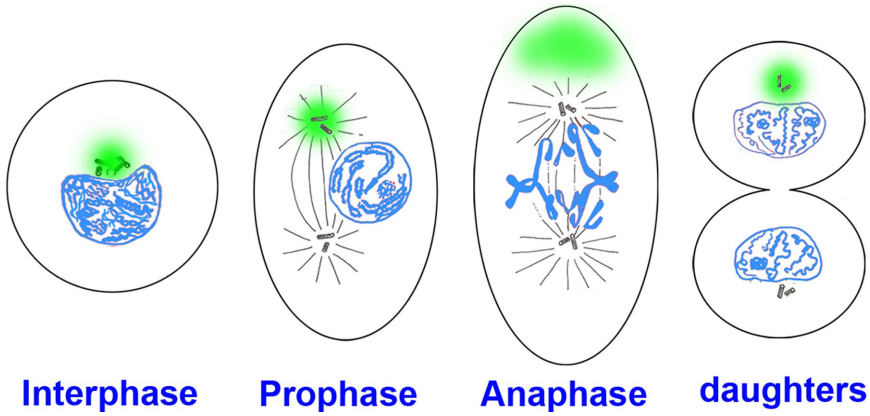

Fig. 5. Cellular model in which the pericentrosomal material (indicated in green) is inherited asymmetrically at mitosis. At interphase, the centrosome and centrioles are surrounded by pericentrosomal material in which proteins destined for degradation concentrate (in this cell the centrosome is located in a nuclear bay cytoplasmic region). During mitosis the centrioles and associated centrosomal MTOC proteins (such as γ-tubulin and pericentrin) divide equally, whereas the pericentrosomal material remains in one pole. We propose that this asymmetry may provide dividing cell populations with a simple physiological mechanism to cleanse themselves of proteins destined for degradation at each cell division.

A Cleansing Mechanism? From a functional point of view, unequal divisions in which proteins targeted for degradation such as pSmad1, phospho-β-catenin, and total polyubiquitinylated proteins are inherited by only one daughter would make excellent sense, for indigestible protein aggresomes have been linked to many protein deposition disorders such as Parkinson's, Alzheimer's, and Huntington's diseases (17, 18). The pericentrosomal localizations reported here in hESCs and Cos7 do not correspond to insoluble pathological aggresomes because they redistribute throughout one daughter cytoplasm during mitosis (Fig. 1). Perhaps proliferating cells that accumulate too much undegraded or undegradable proteins (Fig. 3 *I* and *J*) undergo apoptosis. Thus, dividing cell populations may have a simple physiological mechanism to cleanse themselves of proteins destined for degradation at cell division. If this were the case, nondividing cells such as neurons would be at a disadvantage and more susceptible to disease.

Garbage or Junk? An interesting question for the future is whether the asymmetric proteins are simply "garbage" that the cell does not want to keep or "junk" that might be useful to keep and reuse at later stages. It seems unlikely that such a simple mechanism of partitioning cellular components would not be used for regulatory purposes. Concerning asymmetries involved in taking out the garbage, precedents exist. In *Saccharomyces cerevisiae*, damaged carbonylated proteins have been shown to remain selectively in the mother cell and are not inherited by the bud, providing a possible mechanism of defense to maintain the fitness of the newly born cell (19). Similarly, intestinal crypts of patients with a protein folding disease (neurodegenerative spinocerebellar ataxia type 3) accumulate aggresomes in differentiated crypt cells but not in stem cells (18). Concerning the reuse of asymmetric pericentrosomal materials, during cleavage of the marine snail *Ilyanassa obsoleta* specific mRNAs (encoding regulatory proteins such as Tolloid and Dpp) are associated with one of the sister centrosomes, providing a mechanism for segregating cytoplasmic determinants (20). The cellular mechanism described here could also participate in the asymmetric partition of nuclear β-catenin that occurs during cleavage of nematode and annelid embryos (21, 22).

Centrioles are replicated semiconservatively (2, 23), and during most of interphase the centrosome or cell center from which microtubules radiate remains associated preferentially with the mother centriole (24). Asymmetric cell divisions are well documented during the differentiation of stem cells (25), and in some cases the mother centrosome has been found to associate specifically with the stem cell during asymmetric divisions (26–28). Therefore, the asymmetric inheritance of pericentrosomal materials could potentially influence the outcome of stem cell differentiation decisions.

Another intriguing area of research worth exploring is the possibility that the close proximity between the primary cilium "antenna" whose basal body is formed by the mother centrosome (29, 30) and the proteasomal machinery located in the centrosome (6) might provide an additional layer of regulation in cell signaling.

In conclusion, the unexpected discovery that pericentrosomal asymmetries result in the unequal division of many cellular components (Smad1 and β-catenin targeted for degradation as well as total polyubiquitinylated proteins) opens avenues of research in mitosis. This finding also strengthens the view that the centrosome, the morphological center of the interphase cell, functions as a sophisticated center for the integration of cellular protein catabolism in addition to its role during cell division (2, 7).

Materials and Methods

Cell Culture and Synchronization of Cell Division. The human ES cell line HSF-1 (approved by National Institutes of Health; code UC01) was cultured on feeder cells in 6% CO_2 in 20% knockout serum replacer and knockout DMEM (Gibco) as described (31). hESCs have very poor survival when dissociated as single cells; to study daughter cells we used the recently described method of Sasai and colleagues (10), in which the addition of the ROCK inhibitor Y-27632 (Calbiochem) allows hESC survival. For inhibition of proteasomal enzymatic activity, cells were incubated with 10 μM lactacystin (Calbiochem) for 12 h. To depolymerize microtubules in hESCs, 3 μM nocodazole (Calbiochem) was added for 2 h. Cos7 cells were cultured as described (4). For Cos7 signaling experiments 5 nM BMP7 (R&D) was added in serum-free medium 2 h after synchronization for 1.5 h (4). To analyze daughter cell pairs, Lab-Tek II two-well chamber slides (Nalge/Nunc) were coated with 0.05–0.1% Con A (Sigma), which greatly improves the attachment of daughter cells. For mitotic shake-off cell cycle synchronization, Cos7 Petri dishes were tapped gently 10 times against a vertical surface (11), and the medium was plated immediately into Con A-treated slide chambers.

Antibodies. Primary rabbit antibodies used in this study were: pSmad1^{MAPK} (1:3,500; generated in ref. 4), pSmad1^{GSK3} (1:2,000; ref. 4, antibody A), pSmad1^{Cter} (1:300; Cell Signaling), total Smad1 (1:300; Zymed), anti-*Drosophila* pMad^{MAPK} (1:500; this study, see below), pericentrin (1:1,000; Abcam), and total β-catenin (1:2,500; Sigma). Mouse mAbs used were: phospho-β-catenin (1:500; Ser-33 and Ser-37; Sigma), acetylated-α-tubulin (1:1,000; Sigma), β-tubulin (1:2,000; Sigma), γ-tubulin (1:4,000; Sigma), Oct4 (1:200; Santa Cruz), and polyubiquitin (1:1,000; Biomol). Secondary antibodies included: Alexa 488-conjugated anti-rabbit (1:1,000; Molecular Probes), Cy3-

conjugated anti-rabbit (1:500; Jackson Labs), or Cy3-conjugated anti-mouse IgM or IgG (1:500; Jackson Labs).

Immunostaining of Mammalian Cells. hESCs and Cos7 were grown on two-well chamber slides and fixed in fresh 4% paraformaldehyde for 15 min and permeabilized by treatment with 0.2% Triton X-100 in D-PBS for 10 min. For γ-tubulin staining, an additional step of antigen retrieval was introduced by incubating cells with 0.5% SDS in D-PBS for 5 min (32). After blocking with 5% goat serum and 0.5% BSA in D-PBS for 1 h (blocking solution), primary antibodies were applied overnight at 4°C. Slide chambers were removed and mounted with Vectashield (Vector) containing DAPI stain to visualize DNA.

***Drosophila* Embryo Immunostaining.** A rabbit polyclonal antibody reagent specific for *Drosophila* phospho-Mad^{MAPK} was raised by using the synthetic peptide NSNPNS[PO3]PYDSLAGT by Covance Research Products. Blastoderm-stage *Drosophila* embryos were collected, dechorionated, fixed in formaldehyde, and washed three times with methanol. Embryos were stepwise rehydrated in 0.2% Triton X-100 in PBS and incubated for 1–2 h with gentle rocking, incubated for 1 min in 0.5% SDS, and rinsed in PBS/0.2% Triton X-100 for 5 min, followed by 1-h incubation in blocking solution (PBS/20% goat serum, 2.5% BSA). The SDS treatment serves to make the antigen more accessible (32). For whole-mount embryo immunostaining, the primary antibodies used were rabbit anti-pMad^{MAPK} (1:500; crude antiserum) and monoclonal γ-tubulin (1:500; Sigma), which were incubated overnight in blocking solution at 4°C. Embryos were washed three times for 20 min each by using PBS/0.2% Triton X-100 before applying secondary anti-rabbit Alexa-488-conjugated antibodies (1:1,000; Molecular Probes) and anti-mouse Cy3-conjugated antibodies (1:1,000; Jackson Labs) for 1 h at room temperature. After washing three times with PBS/0.2% Triton X-100, *Drosophila* embryos were mounted on glass slides by using DAPI-containing Vectashield (Vector).

Microscopy. Fluorescent images were photographed with a Zeiss Axiophot or an Axio Imager.Z1 microscope. The Axio Imager.Z1 microscope was equipped with Zeiss ApoTome oscillating grating in the epifluorescence beam, which significantly reduces out-of-focus stray light.

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Supporting Information

Fuentealba *et al*. 10.1073/pnas.0803027105

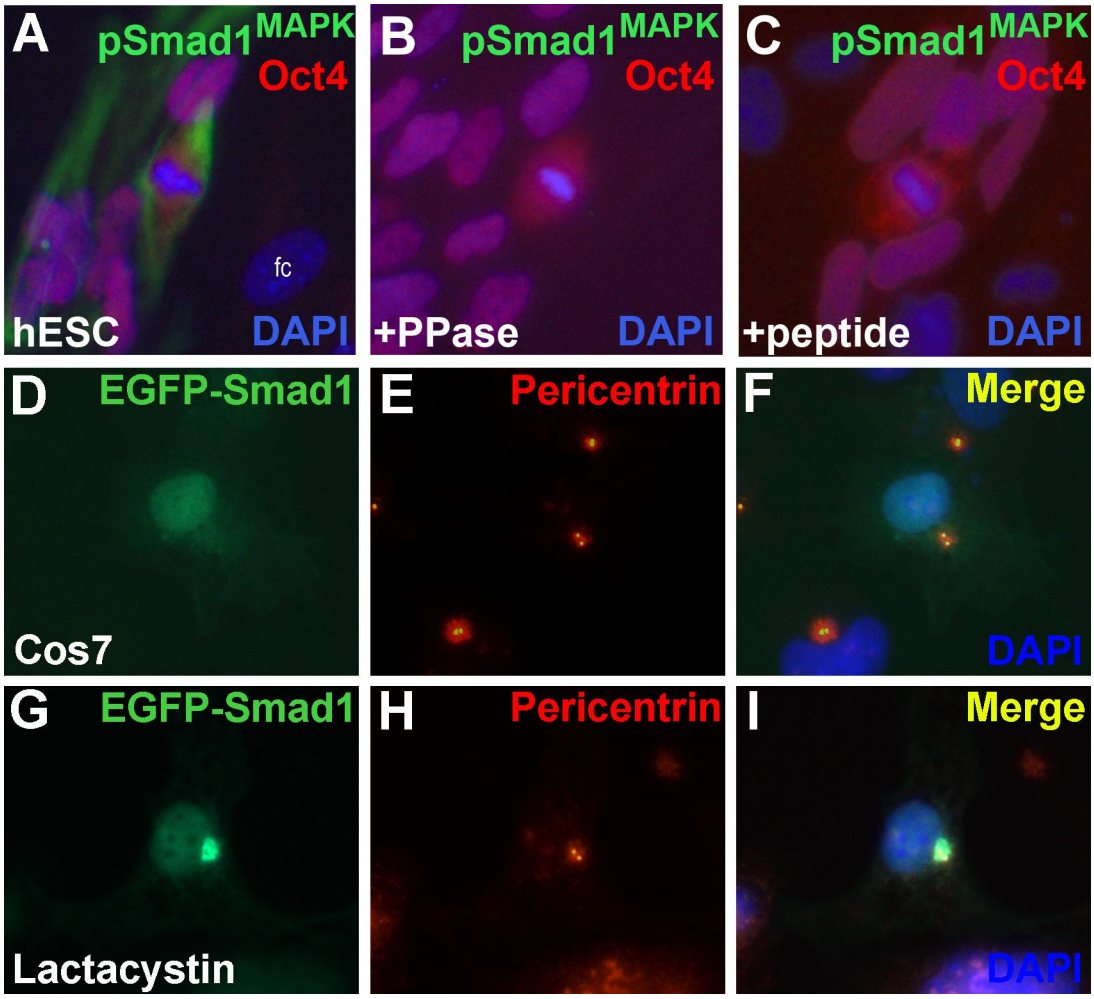

Fig. S1. Specificity of pSmad1 centrosomal localization using antibodies or an EGFP-Smad1 fusion protein to monitor its subcellular localization. (*A*) hESC at metaphase stained with pSmad1^{MAPK} (green) and Oct4 (red). The cell located at the center is at metaphase, and cytoplasmic pSmad1^{MAPK} staining is stronger in the upper half of the cytoplasm. Note that mouse feeder cells (fc) do not stain with this antibody, a fortuitous fact that was very helpful in this study. (*B*) hESC treated with λ phosphatase. Note that the green staining has disappeared and that Oct4 is distributed uniformly throughout the metaphase cytoplasm. (*C*) Staining of pSmad1^{MAPK} is competed by peptide SSDPGS[PO3]PFQMPADT (1 μM). (*D–F*) Localization of EGFP-Smad1 and pericentrin in transfected Cos7 cells cultured in 10% FCS. (*G–I*) In lactacystin-treated Cos7 cells (10 μM for 12 h) EGFP-Smad1 accumulates massively in the peripheral centrosomal region that surrounds the pericentrin-labeled MTOC. This mimics the results obtained by using antibodies in Fig. 2*D*, supporting the view that the normal site of Smad1 degradation is the centrosome. The conclusion that pSmad1^{MAPK} and pSmad1^{GSK3} antigens accumulated in the centrosome depended on immunolocalizations obtained with rabbit antibodies. To exclude any possible artifacts, we analyzed the localization of an EGFP-Smad1 fusion protein previously shown by the Lodish group (1) to shuttle between nucleus and cytoplasm. When cells were treated with the proteasome inhibitor lactacystin, EGFP-Smad1 strongly accumulated in the periphery of the centrosome. We conclude from these experiments, which are independent of any antibody stainings, that Smad1 is indeed transported to centrosomal proteasomes for its degradation. (Magnification: $\times$450.)

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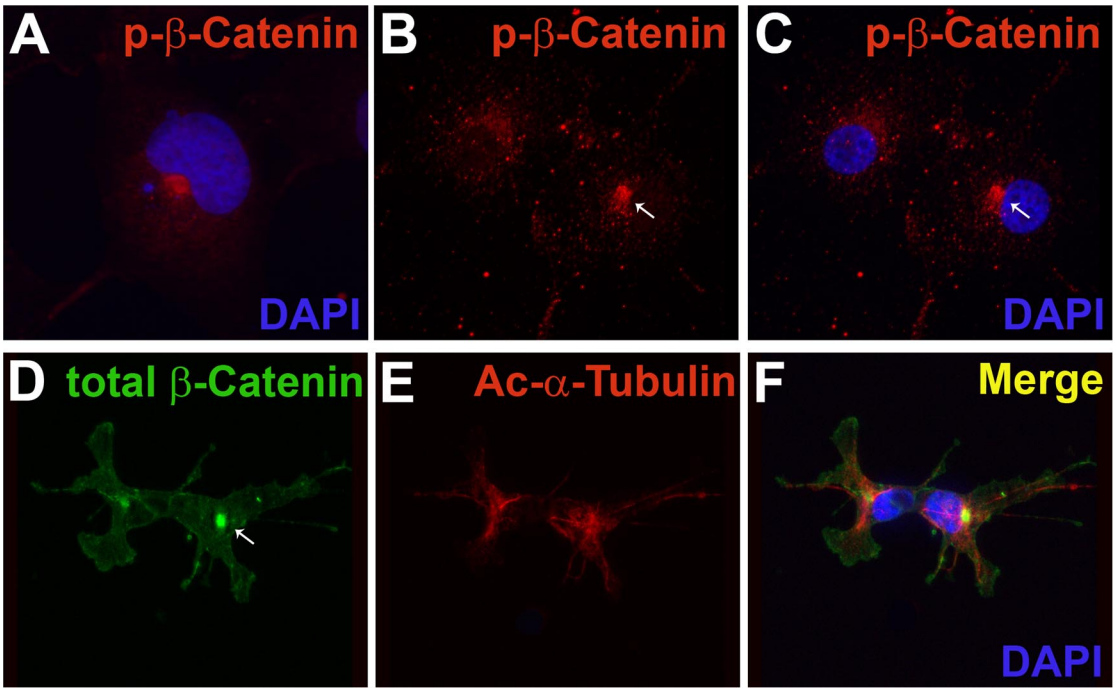

Fig. S2. Asymmetric inheritance of β-catenin phosphorylated by GSK3 in the pericentrosomal region of Cos7 daughter cells. (*A*) Interphase Cos7 cell showing strong phospho-β-catenin staining in the nuclear bay. When a cell displays a nuclear bay, the centrosomes are invariably located within this region of the cytoplasm. (*B*) The phospho-β-catenin mAb asymmetrically marks a juxta-nuclear region in one daughter cell (arrow). (*C*) Asymmetric phospho-β-catenin (arrow) and the position of nuclei stained with DAPI DNA stain are shown. (*D*) Daughter cells stained with anti-total β-catenin antibody in the cell membrane, cytoplasm, and pericentrosomal region. The arrow indicates the strongest region of pericentrosomal staining. (*E*) Acetylated-α-tubulin antibody marks the cellular microtubular network that converges at the centrosome. (*F*) Merged image showing that the strongest β-catenin signal is associated with the centrosomal region. β-Catenin is a Wnt-regulated protein known to be phosphorylated by CK1α and GSK3 enzymes found in a cytoplasmic destruction complex containing Axin and additional proteins (1). After phosphorylation, β-catenin is recognized by the E3 ubiquitin ligase βTrCP, polyubiquitinylated, and degraded in proteasomes (2). Note that the pericentrosomal fraction of β-catenin was stained preferentially by the β-catenin monoclonal phospho-specific antibody directed against phospho-serines 33 and 37. These two residues must be phosphorylated by GSK3 before β-catenin can be recognized by βTrCP (1). These results suggest that the β-catenin destruction machinery is normally located in the pericentrosomal region. (Magnification: ×460.)

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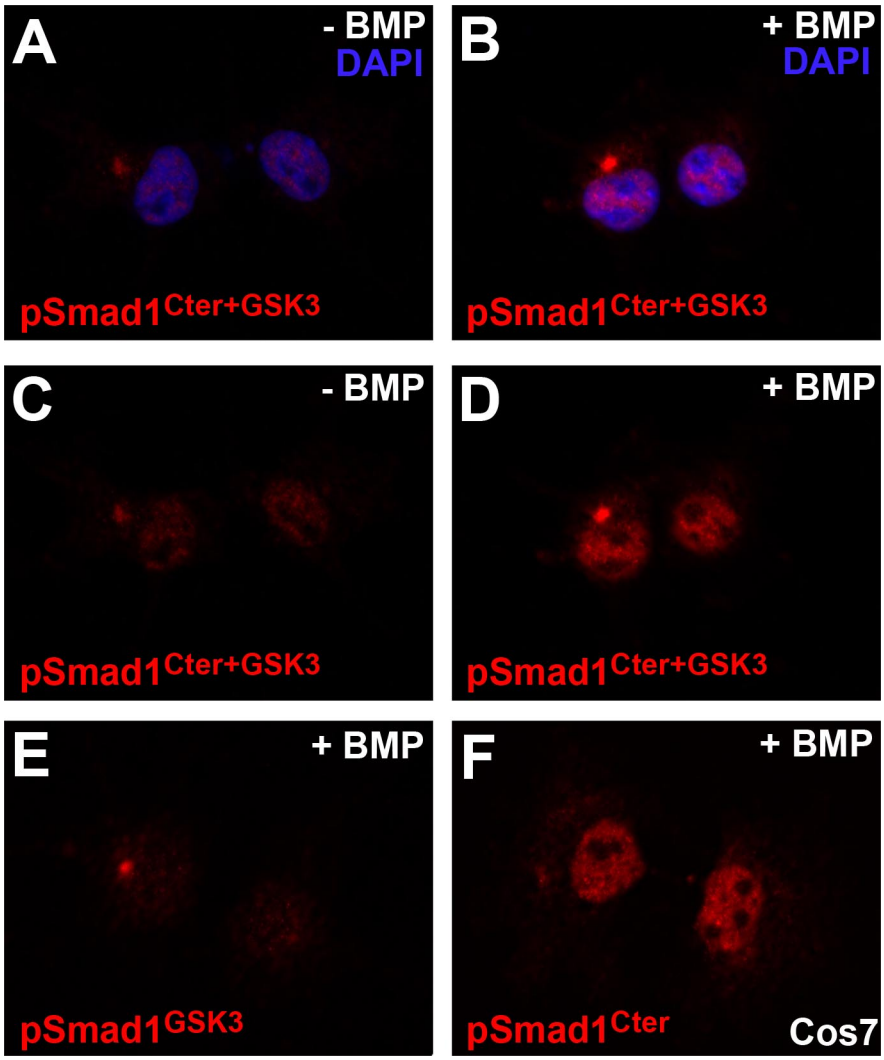

Fig. S3. BMP signaling is not affected in daughter cells displaying pericentrosomal asymmetries. In this experiment we addressed the question of whether the asymmetric distribution of centrosomal material affects the cellular response to BMP signaling. Cos7 synchronized daughter cells were cultured for 1.5 h with or without 5 nM BMP7 and immunostained with a combination of pSmad1^{Cter} and pSmad1^{GSK3} antibodies. (*A* and *C*) Cos7 daughter cells stained both for pSmad1^{Cter} and pSmad1^{GSK3} in red, no BMP added. (*B* and *D*) In BMP7-treated daughter cells the nuclear and centrosomal staining increases. Note that the nuclear pSmad1 staining is equal in both nuclei, reflecting equal BMP signaling levels. *A* and *B* were counterstained for DNA with DAPI. (*E*) Pair of daughter cells stained with pSmad1^{GSK3} alone and treated with BMP. Note that this antibody stains the centrosome strongly and the nucleus very weakly. (*F*) Daughter cell pair stained with pSmad1^{Cter} alone; most of the staining is nuclear and equal between both nuclei. The conclusion we draw from this experiment is that in cell pairs with asymmetrical centrosomes the levels of nuclear pSmad1^{Cter} staining are identical in both daughter cells, which respond equally to the BMP7 signal. The fraction of Smad1 targeted for degradation in the centrosome does not affect the transduction of the BMP7 signal. (Magnification: ×540.)

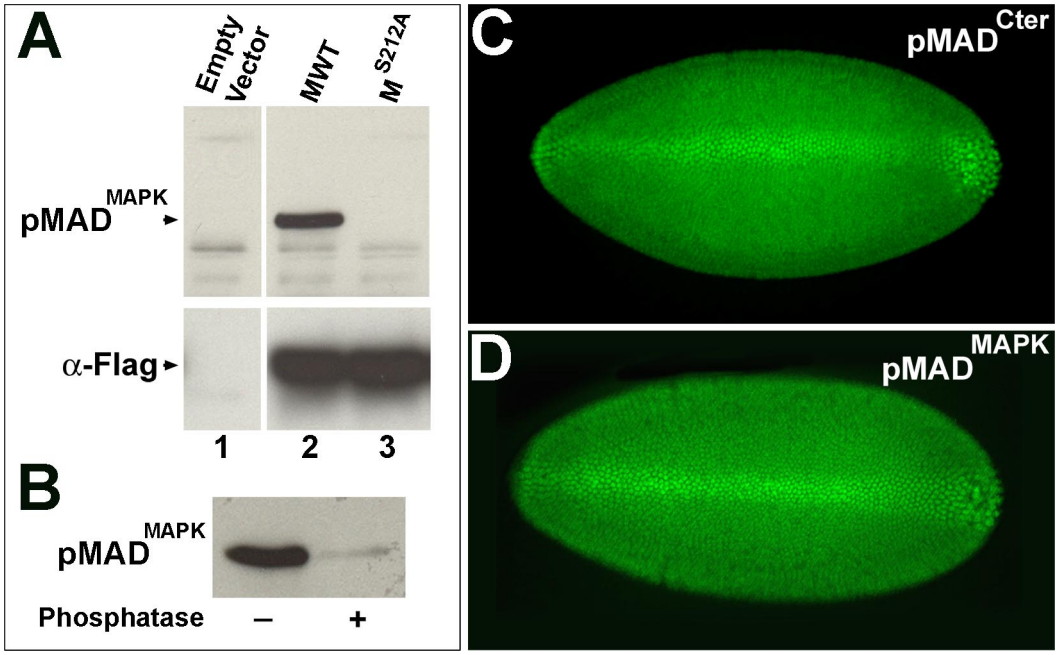

Fig. S4. The pMad^{MAPK} antibody is specific for phosphorylated Ser-212 in *Drosophila*. (*A*) *Drosophila* S2 cells transiently transfected with empty vector pUAS vector, Mad wild-type (MWT) or Mad in which Ser-212 was mutated into Ala (M^{S212A}). Proteins were analyzed by Western blot after induction of Gal4 by the metallothionine promoter (1). Note that MWT protein is expressed in lane 2, but not in S2 cells transfected with vector alone, and that in lane 3 the M^{S212A} mutant is not recognized by the rabbit polyclonal antiserum. The levels of transfected Mad expression were similarly induced, as indicated by a Flag tag introduced at its amino terminus. (*B*) pMad^{MAPK} antigenicity was greatly decreased by treatment with bacteriophage λ phosphatase, confirming that the antibody is phospho-specific. (*C* and *D*) At the early gastrula stage, Mad C-terminal phosphorylation (pMad^{Cter} caused by Dpp receptors) and pMad^{MAPK} displayed very similar staining patterns, with a dorsal stripe, and strong staining in posterior pole germ cells. MAPK-phosphorylation is involved in the termination of Dpp signaling (Fig. 1*A*) and therefore the similarities between pMad^{Cter} and pMad^{MAPK} are in agreement with the predictions of the models proposed in refs. 2 and 3 for vertebrate Smad1. We conclude that the pMad^{MAPK} antiserum, which was not affinity-purified, provides a specific reagent for *Drosophila* studies. An anti-phospho-hSmad1 antibody (1:200 dilution, ref. 4) was used for pMad^{Cter} immunostaining. (Magnification: ×120.)

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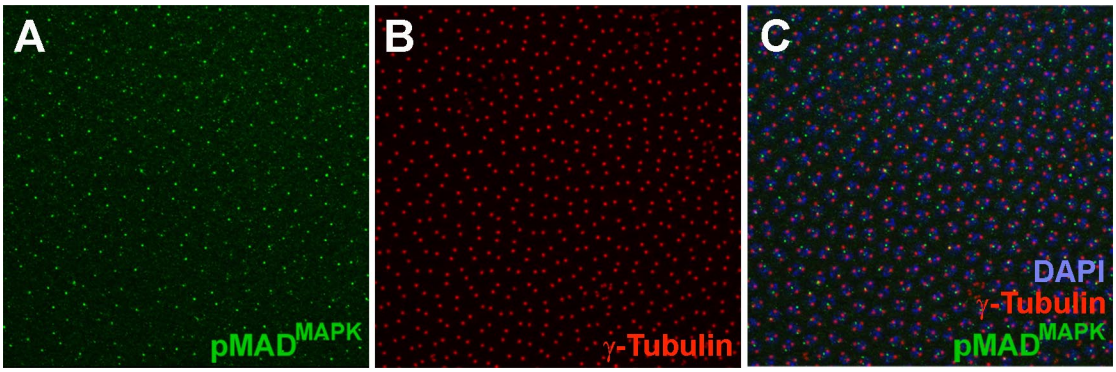

Fig. S5. *Drosophila* embryos at the cellular blastoderm stage contain a single concentrated spot of pMad^{MAPK} antigen near one of the centrosomes marked by γ-tubulin. A rabbit polyclonal antibody reagent specific for *Drosophila* Mad phosphorylated by MAPK at its unique PXSP site was developed (see *Materials and Methods*). (*A*) In the low-power field shown here 445 spots of pMad^{MAPK} were counted. (*B*) Using γ-tubulin, 842 centrosomes were counted in the same field (close to a 2:1 ratio). (*C*) Merged version including DAPI DNA stain in blue. This is in agreement with higher-power photographs (Fig. 4) showing a single spot of pMad^{MAPK} per cell adjoining or nearby one of the centrosomes. This is particularly noticeable in cells at the beginning of the cell cycle, in which the centrosomes have not yet started to separate from each other. All *Drosophila* blastoderm cells had two well defined centrosomes marked by γ-tubulin, except for mitotic cells. Each blastoderm cell has one cytoplasmic pMad^{MAPK} spot adjoining or in the vicinity of only one of the centrosomes. See Fig. 4 for discussion.(Magnification: ×430.)