Regulation of cellular response to oncogenic and oxidative stress by *Seladin-1*

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Expression of multiple oncogenes and inactivation of tumour suppressors is required to transform primary mammalian cells into cancer cells^{1–3}. Activated Ha-RasV12 (Ras) is usually associated with cancer, but it also produces paradoxical premature senescence⁴ in primary cells by inducing reactive oxygen species⁵ followed by accumulation of tumour suppressors p53 and p16^{INK4a} (ref. 4). Here we identify, using a direct genetic screen, *Seladin-1* (also known as *Dhcr24*) as a key mediator of Ras-induced senescence. Following oncogenic and oxidative stress, *Seladin-1* binds p53 amino terminus and displaces E3 ubiquitin ligase Mdm2 from p53, thus resulting in p53 accumulation. Additionally, *Seladin-1* associates with Mdm2 independently of p53, potentially affecting other Mdm2 targets. Ablation of *Seladin-1* causes the bypass of Ras-induced senescence in rodent and human fibroblasts, and allows Ras to transform these cells. Wild-type *Seladin-1*, but not mutants that disrupt its association with either p53 or Mdm2, suppresses the transformed phenotype. The same mutants are also inactive in directing p53-dependent oxidative stress response. These results show an unanticipated role for *Seladin-1*, previously implicated in Alzheimer's disease⁶ and cholesterol metabolism⁷, in integrating cellular response to oncogenic and oxidative stress.

We decided to search for regulators of oncogene-induced senescence using a targeted genetic screen (Fig. 1a) with a genetic suppressor element (GSE)⁸ complementary DNA library prepared from Rat1 cells. We expected that inactivation of genes essential for Ras-induced senescence by GSE sequences would allow cells to escape senescence, continue cell division and eventually form transformed cell colonies in the presence of ectopic expression of an activated Ras allele (Ha-RasV12). By using this screen, we isolated multiple independent antisense GSE sequences corresponding to a broadly expressed oxidoreductase gene (Fig. 1b, Supplementary Fig. 1), initially identified in plants (*DIMINUTO/DWARF1*)⁹ with the human orthologue originally described in neuronal cells (*Seladin-1*)⁶, and later shown to encode 3 β -hydroxysterol- Δ^{24} -reductase activity (*DHCR24*)⁷, further referred to here as *Seladin-1*.

To confirm the role of *Seladin-1* in senescence-inducing Ras signalling, early passage rat embryo fibroblasts (REFs) were infected with retrovirus expressing *Seladin-1* GSE (pBABEpuroR1-4; Supplementary Fig. 1). This resulted in a significant reduction of *Seladin-1* messenger RNA (not shown) and protein levels (up to 90%; Fig. 1c, left panel), with no apparent effect on cell growth or morphology (Fig. 1c, right panel). Upon introduction of Ras through retroviral infection, REF cells co-infected with the control vector (pBABEpuro) were arrested in 6–10 days, as expected⁴. The 'flat' cellular morphology and positive staining of these cells in senescence-associated β -galactosidase (SA- β -gal) assay¹⁰ indicated that they were senescent (Fig. 1d, upper left). In contrast, upon Ras introduction, REF cells with *Seladin-1* knockdown continued to proliferate, exhibited a 'condensed' cell morphology, and were not stained in SA- β -gal assay (Fig. 1d, upper right panel). Furthermore,

Figure 1 Involvement of *Seladin-1* in Ras-induced premature senescence. **a**, A genetic screen for regulators of Ras-induced premature senescence. GSE, genetic suppressor element. **b**, *Seladin-1* expression in adult mouse tissues (northern). GAPDH, loading control. kb, kilobases. sk., skeletal. **c**, Left: western blot of *Seladin-1* protein in rat embryo fibroblasts (REFs) and REFs expressing *Seladin-1* GSE (pBABEpuro-R1-4). Right: SA- β -gal staining of REFs infected with vector pBABEpuro or pBABEpuro-R1-4. **d**, SA- β -gal staining of REFs infected with pBABEpuro or pBABEpuro-R1-4 and transduced with pWZLhygro or pWZLhygro-Ras (upper row); tumorigenicity in nude mice (lower row). **e**, Left: western blot of *Seladin-1* in naive WI38-TERT cells (lane 1), three clones transduced with pBABEpuro-Seladin-1si (lanes 2–4) or pBABEpuro (lanes 5–7). Right: penetrance of the senescent phenotype (SA- β -gal assay). Values are the means+s.d. ($n=3$). **f**, Upper row: SA- β -gal staining of WI38-TERT cells transduced with either empty pBABEpuro (left and right) or pBABEpuro-Seladin-1si (middle) following transduction with pWZLneo-RasV12 (middle and right) or with pWZLneo vector (left). Lower row: anchorage-independent growth in soft agar of U2OS cells (left), WI38-TERT cells transduced with pBABEpuro-Seladin-1si plus pWZLneo-HaRasV12 (middle) and pBABEpuro-Seladin-1si alone (right).

these cells formed tumours within two weeks when inoculated into nude mice (5/5 cases), indicating that they were oncogenically transformed (Fig. 1d, bottom right).

As rodent and human cells behave differently with regards to oncogenic transformation^{4,11}, we investigated whether *Seladin-1* would have a similar effect on Ras-induced senescence in human cells. We used stable RNAi (RNA interference)¹² to silence *Seladin-1* expression in human WI38 fibroblasts that were immortalized by the ectopic telomerase (TERT) expression. Western blot analysis indicated that Seladin-1 protein expression was abrogated in WI38-TERT cells by *Seladin-1* siRNA (small interfering RNA, pBABepuro-Seladin-1si; Fig. 1e, and Supplementary Methods).

When Seladin-1-ablated WI38-TERT cells were transduced with Ras, similar phenotype was observed as in *Seladin-1* knockdown REFs (Fig. 1e, f and Supplementary Results), indicating that *Seladin-1* is involved in regulating Ras-induced transformation and senescence both in human and rodent cells.

Ras-induced senescence is typically associated with activation of tumour suppressors p53, p16^{INK4a} and, in mouse cells, p19^{ARF} (ref. 4, 13). Intriguingly, while Ras-mediated p16^{INK4a} induction was unaffected in Seladin-1-ablated WI38-TERT cells, silencing of *Seladin-1* prevented Ras-induced p53 accumulation (Fig. 2a). p14^{ARF} shows only slight response to oncogenic Ras, which is in line with previous observations^{14,15}. This suggests that Seladin-1 is

Figure 2 Effect of Seladin-1 on cellular response to Ras and oxidative stress.

a, b, Western blot (WB) analysis; **a**, WI38-TERT/pBABepuro and WI38-TERT/pBABepuro-Seladin-1si cells with (+) or without (–) ectopic Ha-RasV12 (Ras) transduction by WZLneoRasV12 retrovirus; **b**, Expression analysis of p53 and Seladin-1 in whole cell extracts (lanes 1 and 2) and Seladin-1 immunoprecipitates (IP; lanes 3–5) in the presence (+) (lanes 2, 4, 5) or absence (–) (lanes 1, 3) of H₂O₂ treatment. Lane 5, cells treated with N-acetylcysteine (NAC). **c**, Effect of Seladin-1 mutants on p53 binding in PA-1 cells. Immunoprecipitates from cells expressing empty pcDNA3 (Vector), wild-type Seladin-1 (WT), mutants N294T/K306N and S419N before (–) and after (+) treatment with H₂O₂, then blotted with p53 antibody. Anti-Flag shows the recombinant Seladin-1 variants expression. **d**, p53 and p21 in WI38-TERT and WI38-TERT expressing pBABepuro-Seladin-1si. H₂O₂ treatment (upper panel), UV irradiation (middle panel), or γ -irradiation (lower panel). **e**, Western blot analysis of p53 levels in naïve WI38-TERT cells or WI38-TERT ectopically expressing Ha-RasV12, in the presence/absence of H₂O₂ and NAC as indicated. **f**, Top panel; anti-p53 western blot after IP with anti-Mdm2 antibodies of

extracts from WI38-TERT cells treated with H₂O₂. Other panels; direct western blot of the same cell extracts with anti-p53, p21, Mdm2 and GAPDH antibodies. **g–i**, Seladin-1/Mdm2 IP-western blot of WI38-TERT (**g**), WI38-TERT and U2OS (**h**), and MEF and p53^{-/-} MEF cells (**i**) following H₂O₂ treatment as indicated. Asterisk in **h** indicates the 60-kDa Mdm2 fragment expressed in U2OS cells. Lower panel in **i** corresponds to same Seladin-1 IP re-blotted with anti-Seladin-1 antibodies. **j**, Immunoblot with anti-Seladin-1 antibodies of anti-Flag IPs (top panel) or direct western blot with anti-Flag antibodies of whole cell extracts obtained following PA-1 cell transfection with the appropriate Flag-tagged p53 mutants. **k**, Homology between the Mdm2 binding region of p53 and the P-box of Seladin-1, and the p53 binding region of Mdm2 and the M-box of Seladin-1. Alignment was obtained using ClustalW²⁷ (<http://www.ebi.ac.uk/clustalw/#>). Up arrowheads, p53 or Seladin-1 mutations inactivating both p53–Seladin-1 and p53–Mdm2 interaction (for p53 mutants) or Seladin-1–Mdm2 interaction (for Seladin-1 mutants). Δ , Deletion of the M-box from Seladin-1. Down arrowhead, S419N Seladin-1 mutant.

an essential mediator of Ras-induced p53 response. Premature senescence initiated by Ras depends on the increase of intracellular reactive oxygen species (ROS)⁵. Accordingly, scavengers of hydrogen peroxide (H₂O₂), a potent ROS producer, rescue Ras-induced senescence. Moreover, sublethal doses of H₂O₂ provoke a senescence-like phenotype in human fibroblasts¹⁶.

On these grounds, we sought to investigate whether Seladin-1 is integrated in Ras-initiated signalling downstream of ROS. Sublethal doses of H₂O₂ rapidly induced p53 and its downstream target, p21^{Cip1/Waf1}, in control cells (Fig. 2d, top panel). In contrast, H₂O₂ treatment did not cause a significant change in p53 and p21^{Cip1/Waf1} protein levels in *Seladin-1* knockdown cells, indicating that Seladin-1 is required for p53-mediated cellular response to ROS.

Moreover, we found that Seladin-1 is upregulated in response to both oxidative stress and Ras activation. Seladin-1 was induced in a time-dependent manner in WI38-TERT cells following H₂O₂ treatment, reaching the maximum level in 10–12 h (Fig. 3c). Similarly, Seladin-1 levels increased in response to Ras expression (Fig. 3d). Intriguingly, we found that Seladin-1 became associated with p53 in

response to both H₂O₂ treatment (Fig. 2b) and Ras activation (Supplementary Fig. 2). In this context, the hydrogen peroxide scavenger N-acetylcysteine (NAC) prevented accumulation of p53 (ref. 5 and Fig. 2e, top panel) and abrogated Seladin-1–p53 interaction (Fig. 2b). This suggests that increases in intracellular ROS trigger p53 and Seladin-1 interaction.

Immunolocalization studies indicated that Seladin-1, normally confined to the perinuclear cytoplasmic region (ref. 6 and Fig. 3a, left column), concentrated in the nuclei after oxidative challenge (Fig. 3a, middle column and Supplementary Fig. 3). Similar changes in Seladin-1 subcellular localization were also observed in WI38-TERT cells expressing a constitutively active Ras allele (Fig. 3a, right column and 3b). Treatment of these cells with NAC inhibited Seladin-1 induction (Fig. 3d) and its nuclear translocation (Fig. 3f, bottom panel), suggesting that Ras-promoted translocation or nuclear retention of Seladin-1 was mediated by ROS signalling.

Seladin-1 levels and localization were mostly unaffected by mild doses of UV or ionizing radiation (IR; here γ -irradiation), indicating that the cellular response to these stresses does not seem

Figure 3 Regulation of Seladin-1 in response to different stimuli.

a, b. Immunofluorescence staining of: **a**, Seladin-1 in WI38-TERT cells (left column), H₂O₂-treated cells (10 h after treatment; middle column) and pWZLneo-Ras-infected cells (10 days after infection; right column). Seladin-1 was stained with rhodamine (red), and nuclei counterstained with DAPI (blue). **b**, Seladin-1 in WI38-TERT cells transduced with Ras. Confocal microscopy on progressive cell sections showing Seladin-1 (Alexa 488, green), DNA (propidium iodide, red) and co-localization, yellow. **c–e**, Western blot analysis of: **c**, Seladin-1 in WI38-TERT cells before and after H₂O₂ treatment. **d**, Seladin-1

in WI38-TERT cells transduced with pWZLneo (lane 1), pWZLneo-Ras alone (lane 2) or together with NAC treatment (lane 3), naïve WI38-TERT cells (lane 4), WI38-TERT cells treated with H₂O₂ (lane 5) and with H₂O₂ and NAC together (lane 6) as in Fig. 2c.

e, Seladin-1 in WI38-TERT cells before (0 h) and after γ -irradiation (0.5–48 h).

f, Immunofluorescence staining of Seladin-1 in WI38-TERT cells before and 12 h after γ -irradiation (upper), and in WI38-TERT/pWZLneo-Ras cells without and with NAC treatment (lower). Scale bars, 10 μ m. GAPDH, loading control.

to require Seladin-1 (Fig. 3e and f). Accordingly, Seladin-1 ablation failed to prevent UV- and IR-induced p53-dependent growth arrest (Figs 2d, 4e). Taken together, these results support a role of Seladin-1 specifically in the p53 response to oncogenic and oxidative stress which might be determined by the nature of damage caused by different stresses^{17,18}.

To gain further insight into the mechanism of the Seladin-1 effect on p53, we prepared p53 mutants and tested their interaction with Seladin-1 in co-immunoprecipitation (IP) assays. Interestingly, N-terminal p53 mutant(s), deficient in p53–Mdm2 interaction, also lost the ability to bind Seladin-1, suggesting that Seladin-1 associates with p53 at the Mdm2 binding site of the latter (Fig. 2j). In contrast, mutants of the p53 DNA binding domain retain interaction with Seladin-1 (Fig. 2j). Surprisingly, we also observed an association between Seladin-1 and Mdm2 (Fig. 2g–i) that was more pronounced in extracts from cells subjected to oxidative stress (Fig. 2g–i). Moreover, Seladin-1–Mdm2 interaction was independent of p53 and p14^{ARF}, since it was observed also in p53 null (MEF p53^{−/−}) and p14^{ARF} null cells (U2OS) under similar conditions (Fig. 2h, i). These results suggest that Seladin-1 might affect p53 by interfering with Mdm2-mediated p53 regulation.

To test this hypothesis, we prepared bacterially expressed

Flag-p53, Seladin-1 and Mdm2 (Supplementary Fig. 7) and found that, first, Seladin-1 binds p53 and displaces Mdm2 from p53 *in vitro* (Fig. 4c). Second, recombinant Seladin-1 inhibits *in vitro* ubiquitination of Flag-p53 by Mdm2 (Fig. 4d). Finally, levels of Mdm2-associated p53 decrease during oxidative stress *in vivo* (Fig. 2f, top panel) despite concomitant increase in overall p53 and stable Mdm2 levels in the same experiments (Fig. 2f).

Sequence analysis revealed that Seladin-1 contains two adjacent regions of homology with the Mdm2-binding site on p53 (Box P) and with the p53-binding site on Mdm2 (Box M; Fig. 2k and Supplementary Fig. 5). The significance of these sites for Seladin-1–p53 and Seladin-1–Mdm2 interactions is underlined by failure of the appropriate Seladin-1 mutants to interact with p53 (Figs 2c, 4b) and Mdm2 (Supplementary Fig. 6), respectively. Additionally, the Seladin-1 N294T/K306N double mutation, which inactivates its intrinsic DHCR24 activity⁷, has no effect on Seladin-1–p53 interaction (Fig. 2c). Our data support a model where Seladin-1 directly inhibits Mdm2–p53 interaction following oxidative stress, causing a decrease in Mdm2-dependent p53 ubiquitination and degradation, and resulting in accumulation of active p53 and subsequent initiation of the cell cycle arrest. Thus, Seladin-1 shows features of a potential tumour suppressor involved in cellular response to

Figure 4 Seladin-1 as an inhibitor of p53–Mdm2 interaction, and as a potential tumour suppressor. **a**, Effect of Seladin-1 on REF transformation by p53(R175H) and Ha-RasV12. Normalized numbers of transformed foci for vector, wild-type Seladin-1 (Sel-1), and mutant Seladin-1 (Sel-1(S419N); Sel-1(N294T/K306N); Sel-1(L210Q/F211S) and Sel-1(ΔM) (as in Fig. 2k) are shown. The values were normalized to the number of foci observed on the control vector plates. The columns in the graph represent normalized average + s.d. ($n=3$). **b**, Effect of Seladin-1 mutations on its interaction with p53. Top panel, IP with anti-Flag, followed by immunoblot with anti-p53. Bottom panel, Flag immunoblot. P-box Seladin-1 mutant still binds p53. **c**, Inhibition of p53–Mdm2 interaction by the recombinant Seladin-1. Top row, immunoblot of Flag-p53-bound

Mdm2 following incubation with increasing amounts of GST-Seladin-1. Second row, p53-associated Seladin-1 from the same experiment detected with anti-Seladin-1 antibody. Bottom two rows, direct immunoblot of total Flag-p53 or Mdm2 at the conclusion of the experiment. **d**, Inhibition of Mdm2 ubiquitination of p53 *in vitro* by Seladin-1. Asterisk, unspecific band on top of p53. **e**, Effect of H₂O₂ treatment on DNA replication (BrdU incorporation, upper graph) and on senescence induction (SA-β-gal staining, lower graph) in WI38-TERT cells and cells expressing Seladin-1 siRNA (Seladin-1si). Results are mean percentages + s.d. ($n=3$). **f**, Model of Ras- and oxidative stress-initiated signalling pathway. H, human; M, mouse.

Ras/p53-mediated oncogenic signalling and oxidative stress.

Hence, Seladin-1 suppresses oncogenic foci formation promoted by co-expression of dominant negative p53 and oncogenic Ras in REFs (Fig. 4a). This activity depends on p53 and Mdm2 binding, since both M-box and P-box Seladin-1 mutants were ineffective (Fig. 4a, b; Fig. 2c and Supplementary Fig. 6). In contrast, abrogation of DHCR24 activity (N294T/K306N double mutant) had little effect on growth suppression (Fig. 4a) and p53 accumulation following oxidative challenge (Supplementary Fig. 4).

Inactivation of either p53 or p19^{ARF} in combination with an activated Ras allele suffices to transform rodent primary cells. In contrast, p53 mutation fails to overcome Ras-induced senescence in human primary cells^{4,19}. We observed that Seladin-1 ablation uniquely prevents human fibroblasts from arresting in response to Ras, while maintaining Ras growth stimulatory potential. This suggests that in addition to p53, Seladin-1 might affect other pathways relevant to Ras-mediated premature senescence in human cells. Accordingly, since Seladin-1 also interacts with Mdm2 independently of p53, this suggests a possible effect on other Mdm2 targets, such as pRB^{20–24} (Fig. 4f). Additionally, since Seladin-1 displaces Mdm2 from p53, other p53 modifications catalysed by Mdm2²⁵ might also be affected by Seladin-1.

The similarity of the Seladin-1 response to Ras and oxidative stress suggests that Seladin-1 may unexpectedly serve as an important ROS effector in mammalian cells. Concordantly, we observed that cells with Seladin-1 knockdown continued to show BrdU incorporation (Fig. 4e, top panel), cell proliferation (Supplementary Fig. 8) and approximately fourfold less SA- β -gal staining (Fig. 4e, lower panel) after H₂O₂ treatment, suggesting an essential role for Seladin-1 in the oxidative stress response senescence program¹⁶. Interestingly, Ras activation and ROS generation were not affected by Seladin-1 (Supplementary Fig. 9), placing Seladin-1 downstream of Ras and ROS in premature senescence and oxidative stress response pathways (Fig. 4f).

Seladin-1 downregulation coincides with Alzheimer's disease⁶, but the molecular basis of this correlation remains unclear. The only biological activity of Seladin-1 described previously was 3 β -hydroxysterol- Δ ²⁴-reductase (DHCR24) that catalyses the reduction of desmosterol, the last step in cholesterol biosynthesis⁷. We show here that the DHCR24 activity of Seladin-1 is apparently not required for p53 binding and p53-dependent oxidative stress response (Fig. 2c and Supplementary Fig. 4). Nevertheless, two seemingly independent Seladin-1 functions affecting cholesterol and p53 might share some regulatory mechanisms. In fact, oxidation of cholesterol produces ROS²⁶ and this gives rise to a potential crosstalk between these two pathways, with Seladin-1 acting as a potential sensor and/or effector of oxidative stress.

Ras and tumour suppressors p53 and p14^{ARF}/p19^{ARF} are involved in oncogenesis, cellular senescence and ageing. Additionally, both oxidative stress and cholesterol metabolism have been implicated in ageing and degenerative disorders at the organismal level. Our data support the notion that Seladin-1 stands at the intersection of all these processes. □

Methods

Vectors and cell culture

Retroviral vectors pBABEpuro, pWZLhygro and pWZLneo were used to construct pBABEpuro-Seladin-1, pBABEpuro-R1-4 and pBABEpuro-Seladin-1si, pWZLhygro-H-rasV12 and pWZLneo-H-rasV12. pWZLhygro-hTERT was a gift from R. Weinberg. Retrovirus preparation, infection and selection were done as described⁴. Cell lines and growth conditions are described in Supplementary Methods.

GSE cDNA library screen

The retroviral GSE screen was performed as previously described⁸; details are given in Supplementary Methods. Antisense GSE sequences recovered from the screen were used to search against available NCBI databases to find the corresponding genes and their homologues in different species. Accession numbers in GenBank for the corresponding genes are: human *Seladin-1/DHCR24* cDNA D13463 (partial), AF261758 (full); genomic

clone AC009946; mouse cDNA AY039762; *Arabidopsis thaliana* DWF1 U12400; *Caenorhabditis elegans* homologue AF026214.

Senescence-associated β -galactosidase staining

The staining was performed essentially as described⁴. Cells were washed briefly with distilled water after staining and then examined under an inverted Olympus IX70 microscope.

Immunoprecipitation, western blot, cell growth and tumorigenesis

Cells were treated as described in Supplementary Methods.

siRNA inhibition of Seladin-1 expression

Two oligonucleotides were synthesized and annealed: 5'-GATCCCTCGGAAGGAGCA GGGTAGCATTCAGAGATGCTACCTGCTCTTCCA TTTTGGAAA-3' and 5'-AGCTTTTCCAAAAATGGAAGGAGC AGGGTAGCATCTCTTGAATGCTACCTGCTCTTCCAGGG-3' (siRNA target recognition sequence is underlined). The annealed double-strand oligonucleotide was then inserted into the *Bgl*II-*Hind*III sites of pSUPER¹². To improve the RNAi efficiency, the siRNA expression cassette (*Bam*HI-*Sal*I fragment) was removed from pSUPER-Seladin-1si and cloned into *Bam*HI-*Sal*I digested pBABEpuro. The resultant pBABEpuro-Seladin-1si was used to generate retrovirus and infect WI38-TERT cells. Puromycin selection was applied for three days to generate cells with a stable inhibition of Seladin-1.

Seladin-1, mutants, expression, purification, ubiquitination assays

Detailed procedures are described in Supplementary Methods.

BrdU incorporation assay

The assay was done using the *in situ* Cell Proliferation Kit, FLUOS, following the instructions (Roche). Cells were cultured on coverslips, and BrdU labelling was for 4 h. The BrdU signal was examined under an inverted Olympus IX70 microscope equipped with proper fluorescence filters.

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Poly(ADP-ribose) is required for spindle assembly and structure

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The mitotic spindle is typically thought of as an array of microtubules, microtubule-associated proteins and motors that self-organizes to align and segregate chromosomes¹. The major spindle components consist of proteins and DNA, the primary structural elements of the spindle¹. Other macromolecules including RNA and lipids also associate with spindles, but their spindle function, if any, is unknown. Poly(ADP-ribose) (PAR) is a large, branched, negatively charged polymeric macromolecule whose polymerization onto acceptor proteins is catalysed by a family of poly(ADP-ribose) polymerases (PARPs)². Several PARPs localize to the spindle in vertebrate cells, suggesting that PARPs and/or PAR have a role in spindle function². Here we show that PAR is enriched in the spindle and is required for spindle function—PAR hydrolysis or perturbation leads to rapid disruption of spindle structure, and hydrolysis during spindle assembly blocks the formation of bipolar spindles. PAR exhibits localization dynamics that differ from known spindle proteins and are consistent with a low rate of turnover in the spindle. Thus, PAR is a non-proteinaceous, non-chromosomal component of the spindle required for bipolar spindle assembly and function.

PAR polymers are the product of post-translational modifications and are required for normal cell division; in *Drosophila*, PARP knockouts are embryonic lethal³. PAR is polymerized by PARPs onto acceptor proteins using NAD⁺ as substrate², and the concentration, length and extent of PAR branching are regulated by a balance of activities of the PARPs and poly(ADP-ribose) glycohydrolase (PARG), a highly specific, processive endo- and exoglycosidase⁴. Most PARPs localize to the spindle: PARP1 and PARP2 localize to centromeres and interact with CENPA, CENPB and Bub3 (ref. 5); VPARP localizes throughout the spindle⁶; PARP1 and PARP3 associate with centrosomes²; and tankyrase1 and 2 are found at chromosomes and spindle poles⁷, where they interact with the spindle pole protein NuMA⁸. Cellular PAR concentrations

markedly increase during metaphase and anaphase–telophase owing to PARP activity⁹. Together, these observations suggest that PARPs, or PAR itself, might be important in spindle function.

We examined PAR localization in metaphase spindles by staining isolated spindles—assembled in cycled *Xenopus* egg extract and in somatic cells—using three different antibodies against PAR, and varied fixation conditions (Fig. 1a, b). In all cases, PAR was filamentous and punctate in appearance, co-localized with spindle microtubules, and was enriched at spindle poles and kinetochores (Fig. 1a, b; see also Supplementary Fig. S1). PAR filaments were most evident without fixation and became more punctate after fixation, suggesting that traditional fixation methods developed for proteins perturb PAR localization. PAR localization to the kinetochore is particularly interesting in light of data demonstrating PARP1 recruitment to metaphase centromeres¹⁰, suggesting a role for PARP1 or PAR in centromere/kinetochore function.

The staining pattern in metaphase cells was similar to isolated *Xenopus* spindles except for a more pronounced enrichment at the poles (Fig. 1b). At the poles, PAR was primarily associated with spindle pole material rather than centrosomes, and spindle pole staining was similar to the localization pattern seen for tankyrase1 (ref. 11). Interestingly, PARG also co-localized with spindle microtubules in BSC1 and HeLa cells (Fig. 1c). This co-localization of PARPs, PAR and PARG with spindle microtubules suggests that one or more PARP(s) and PARG may actively regulate PAR levels in the spindle.

To determine whether PAR is enriched in the metaphase spindle, we compared the amount of spindle-associated PAR to total mitotic frog egg extract (CSF) by isolating spindles from *Xenopus* extract, and resolving equivalent concentrations of purified spindles and CSF on SDS polyacrylamide gels. Immunoblotting with anti-PAR antibodies showed a ~10-fold enrichment of PAR in the spindle relative to surrounding cytoplasm (Figs 1d and 2c). Most of the poly(ADP-ribosyl)ated (PARsylated) proteins were between ~97 and ~200 kDa. These may represent a single, highly modified protein, or a population of PARsylated proteins of varying sizes (Figs 1d and 2c). PAR and tubulin concentrations were unaffected by detergent treatment before spindle isolation, showing that PAR directly associates with the spindle rather than the membranes that co-purify with isolated spindles.

To ask whether PAR has an important function in the spindle, we assembled spindles in the presence of X-rhodamine-labelled tubulin in extracts containing replicated chromosomes¹² (cycled spindles), and perturbed PAR polymer by adding exogenous PARG or high concentrations of purified anti-PAR antibodies. We observed spindle structure using real-time, spinning-disk confocal microscopy (Fig. 2a) or examined fixed samples at defined time points (Fig. 2b, e). Notably, addition of 100 µg ml⁻¹ PARG resulted in the rapid breakdown of spindle structure (Fig. 2a; see also Supplementary Movie M1). Microtubules rapidly splayed outward from the spindle centre, after which the two half spindles appeared to become disconnected and the degree of anti-parallel microtubule overlap decreased markedly. This effect was specific to the hydrolytic activity of PARG, as simultaneous addition of 100 µM of the PARG inhibitor ADP-HPD¹³ blocked the phenotypic effect of PARG (Fig. 2b). We quantified the effect of PARG addition on both PAR and tubulin concentrations in the spindle by isolating treated and untreated spindles and blotting for PAR or tubulin (Fig. 2c). Addition of 100 µg ml⁻¹ PARG to assembled spindle reactions resulted in the complete hydrolysis of PAR polymer up to the detection limit of our testing, whereas simultaneous addition of 100 µM ADP-HPD partially protected PAR, especially at high molecular weight. Addition of 500 µg ml⁻¹ of affinity-purified anti-PAR antibody to cycled spindles phenocopied the effects of PARG addition (Fig. 2a; see also Supplementary Movie M2). In all cases extracts remained in mitosis, as demonstrated by the presence of condensed chromosomes. The phenotype of PAR hydrolysis was not