Aurora-A recruitment and centrosomal maturation are regulated by a Golgi-activated pool of Src during G2

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The Golgi apparatus is composed of stacks of cisternae laterally connected by tubules to form a ribbon-like structure. At the onset of mitosis, the Golgi ribbon is broken down into discrete stacks, which then undergo further fragmentation. This ribbon cleavage is required for G2/M transition, which thus indicates that a ‘Golgi mitotic checkpoint’ couples Golgi inheritance with cell cycle transition. We previously showed that the Golgi-checkpoint regulates the centrosomal recruitment of the mitotic kinase Aurora-A; however, how the Golgi unlinking regulates this recruitment was unknown. Here we show that, in G2, Aurora-A recruitment is promoted by activated Src at the Golgi. Our data provide evidence that Src and Aurora-A interact upon Golgi ribbon fragmentation; Src phosphorylates Aurora-A at tyrosine 148 and this specific phosphorylation is required for Aurora-A localization at the centrosomes. This process, pivotal for centrosome maturation, is a fundamental prerequisite for proper spindle formation and chromosome segregation.
The Golgi apparatus is a major station of the secretory pathway that processes and sorts newly synthesised proteins and lipids during the transport from the endoplasmic reticulum (ER) to the plasma membrane or to the endo-lysosomal system. The Golgi apparatus has been also defined as a ‘carbohydrate factory’, for the glycosylation and other post-translational modifications of proteins and lipids, and, finally, it acts as a signalling platform onto which diverse signalling, sorting and cytoskeleton proteins can localize and function. Structurally, the Golgi apparatus is composed of stacks of flat cisternae that are laterally connected by membranous tubular ‘bridges’ to form a continuous system, which is referred to as the Golgi ribbon. In mammals, the mitotic inheritance of the Golgi apparatus involves its progressive fragmentation, which is a process that can be schematically divided into three main steps: (i) the breakup of the Golgi ribbon into stacks (referred to as unlinking), which occurs in G2-phase and is detectable only using high-resolution imaging techniques; (ii) the fragmentation of these stacks into cisternae (referred to as unstacking), which occurs in prophase; and (iii) finally, the further fragmentation into dispersed fragments and vesicles. Once the dispersed Golgi membranes have been divided between the daughter cells during telophase, they can then reassemble and fuse into a new fully functional Golgi ribbon.

Importantly, inhibition of Golgi ribbon unlinking results in G2-arrest of the cell cycle or, in delay of G2/M transition, depending on the experimental approach. This indicates that a Golgi mitotic checkpoint controls the correct inheritance of this organelle. We have previously addressed the functional connection between Golgi fragmentation and cell cycle regulation and we have shown that a block in this Golgi unlinking results in a reduced recruitment of Aurora-A kinase to the centrosomes and its impaired activation at the centrosomes during the G2-phase of the cell cycle. Aurora-A has a pivotal role in many centrosome-based processes. Indeed, a complex network of regulatory systems ensures an ordered and spatially restricted recruitment of Aurora-A to the centrosome. We show that Src is activated at the centrosome and we have shown that a block in this Golgi unlinking results in inhibition of Aurora-A at the centrosome. This inhibition was not the consequence of the delayed G2/M transition caused by Src inhibition, as we fixed the samples at a time when most of the cells were in G2 and were positive for Aurora-A at the centrosome.

Src overactivation is known to induce Golgi fragmentation by stimulating dynamin-dependent post-Golgi traffic. Thus, we asked whether Src inhibition could also prevent the G2-specific Golgi unlinking, as a block of this process can indirectly result in a reduction of Aurora-A recruitment. HeLa cells were either accumulated in S-phase (T-block) or in G2-phase (G2-arrested). These G2-arrested HeLa cells were also treated with U0126, to inhibit MEK activity that is required for the severing of the Golgi ribbon, and with SU6656, to test the requirement for Src in fragmentation of the Golgi ribbon. The cells were then fixed and processed for confocal microscopy to analyse the extent of the ribbon unlinking.

In S-phase-enriched cells, the Golgi membranes were composed of a average of four Golgi objects (Fig. 1c,d, T-block, lane 1), as previously described; instead, in G2-arrested cells, the Golgi complex was composed of about 9 objects (Fig. 1c,d, G2-arrested, lane 2), as expected for G2-arrested cells. Inhibition of MEK in G2-blocked cells resulted in a decrease in the extent of fragmentation, in line with previous results. Conversely, inhibition of Src did not reduce Golgi fragmentation. Thus, we further tested the role of Src in mitotic Golgi fragmentation using an in vitro assay. In permeabilized NRK cells incubated with interphase cytosol, the Golgi remained compact and perinuclear. Conversely, in cells incubated with the mitotic cytosol, the Golgi apparatus was fragmented and dispersed. Src inhibition in mitotic extracts using SU6656 did not cause any reduction in the fraction of cells with fragmented Golgi.
Figure 1 | Src-dependent Aurora-A recruitment is a process downstream the Golgi ribbon fragmentation. (a) HeLa cells grown on coverslips and arrested in S-phase using double-thymidine block. Two hours before fixing, cells were incubated with 10 μM SU6656 or PP2 or DMSO. After thymidine washout, cells were processed for immunofluorescence analysis with anti-Aurora-A and anti-γ-tubulin antibodies. Graph is the quantification of the percentage of cells positive for Aurora-A at the centrosome as means (± s.d.) from three independent experiments (n = 200). Two-tailed Student t-test was applied to the data (P value < 0.05). (b) Representative images of quantified cells as in a. Scale bar, 3 μm. (c,d) HeLa cells arrested in S-phase with double-thymidine block (T-block) or accumulated in G2 with bis-benzimide for 18 h (G2-arrested), respectively. Where indicated, cells were treated with 80 μM MEK inhibitor (U0126) or DMSO (6 h) or 10 μM SU6656 (2 h). (c) Representative images show Hoechst 33342-labelled DNA (blue) and the Golgi complex (red) stained with anti-GM130 antibody. For qualitative analysis of the Golgi, the GM130 staining was inverted using ‘Invert’ function of the ImageJ software package. Scale bar, 5 μm. (d) Quantification of Golgi linking in the populations in c with ImageJ software to apply a threshold and count the above-threshold fluorescent ‘Golgi objects’, using the ‘Analyse Particle’ function. The analysed cells (n = 50) were chosen randomly. Data are mean values (± s.e.m.) from three independent experiments. Two-tailed Student’s t-tests were applied to the data (P value < 0.05). (e,f) NRK cells grown on coverslips and arrested in S-phase with 2.5 μg ml−1 aphidicolin. After aphidicolin washout, cells were washed for cell cycle progression. Src inhibitor SU6656 was added to the mitotic cytosol before the permeabilization procedure and cells were incubated with mitotic cytosol with SU6656 or DMSO. The Golgi apparatus was labelled with anti-giantin antibody (red). (e) Representative images of the cells labelled with anti-giantin (red) for the Golgi and with Hoechst 33342 (blue) for the cell cycle phase. Scale bar, 5 μm. (f) Quantification of the extent of fragmentation of the Golgi measured by counting the number of cells (n = 20) in which the Golgi apparatus was fragmented/dispersed. Data are mean values (± s.d.) from three independent experiments, each carried out in duplicate. Two-tailed Student’s t-tests were applied to the data (P value < 0.0001). DMSO, dimethylsulphoxide.

(fragmentation index; Fig. 1f, mitotic cytosol + SU6656), thus confirming that Src is not involved in G2-ribbon unlinking and mitotic fragmentation.

Golgi fragmentation in G2 induces activation of Src. As Src inhibition induces a delay in the G2/M progression14 (Supplementary Fig. 2b,c) and an impairment of the recruitment of Aurora-A to the centrosome (Fig. 1a), without affecting the structure of the Golgi apparatus (Fig. 1c,d), one possibility is that a Src-based signalling pathway acts downstream of the Golgi ribbon breakup, which then leads to the recruitment of Aurora-A.
If this is the case, the fragmentation of the Golgi ribbon during G2 should induce the activation of the kinase Src, which can be monitored by the phosphorylation level of Tyr416 (pY416-Src). Src has been reported to be located not only at the level of the plasma membrane but also in other cellular compartments, including the Golgi apparatus18. Synchronized HeLa cells were fixed in coincidence with the thymidine washout (S-phase blocked) or 8 h later (G2-phase). Some of the cells were also treated with the JNK inhibitor VIII (JNKi-VIII), to prevent Golgi blocked) or 8 h later (G2-phase). Some of the cells were also fixed in coincidence with the thymidine washout (S-phase blocked). To reveal active Src because it was reduced by the Src inhibitor PP2 (Fig. 2b), as this condition induces bypass of the Golgi checkpoint9. When the Golgi complex was in the form of isolated stacks, the JNKi-VIII did not reduce the levels of active Src (Fig. 2b), thus suggesting that Src is specifically activated by the unlinking process. Importantly, a block of G2-specific ribbon unlinking by treatment with the JNK inhibitor VIII (JNKi-VIII) strongly reduced the activation of Src at the Golgi (Fig. 2b). To determine whether the activation of Src is directly regulated by Golgi unlinking, the Golgi apparatus was forced to be in the form of isolated stacks through siRNA-mediated GM130 depletion22 (Fig. 2b), as this condition originates from the TGN. Moreover, we have employed the proximity ligation assay (PLA) to test the association of Src with Golgi markers. Using this approach, interactions between two proteins result in the formation of fluorescent dots in fixed cells23. As shown in Supplementary Fig. 3c-f, Src appears to be located at the Golgi complex in association with α-mannosidase-II (ref. 24), independently of the cell cycle phase, thus suggesting that its activation, and not its recruitment, is the step regulated by Golgi fragmentation. These results indicated that the Golgi fragmentation correlates with the activation of Src at the Golgi apparatus. To address this aspect by an independent approach, we monitored the activation of Src by western blotting of total cell lysates using the anti-pY416-Src antibody. To induce Golgi fragmentation, HeLa cells were synchronized in S-phase and treated for a time from 5 to 120 min with brefeldin-A (BFA), a fungal toxin that causes disassembly of the Golgi apparatus25. The cells were then processed for both confocal analysis, to monitor the Golgi structure and western blotting, to verify Src activation. Under these conditions, a short treatment with BFA resulted in the loss of the ribbon-like organization of the Golgi (Fig. 2c), and this correlated with Src activation, as revealed by western blotting (Fig. 2d,e). Activation of Src peaked after 5 min incubation with BFA, and was specifically inhibited by the Src inhibitor PP2 (Fig. 2d,e). After 30 min of incubation with BFA, the Golgi apparatus is completely dissolved and redistributed into the ER26. Thus, it is plausible that the machinery responsible for Src activation has been disrupted after this time point, resulting in basal levels of active Src (Fig. 2e).

Altogether, these data suggest that the Golgi apparatus acts as a signalling hub to monitor Golgi unlinking and to induce compartmentalized Src activation27, which could be an important determinant in functionally linking the Golgi breakdown to Aurora-A.

**Golgi unlinking induces Src-Aurora-A interaction.** We next examined whether Golgi-dependent Src activation and Src-dependent Aurora-A recruitment are functionally linked. Thus, HeLa cells enriched in the G2-phase were co-transfected with vectors for the expression of Aurora-A-GFP and the untagged versions of the wild-type (wt), constitutively active (Y527F) and kinase-dead (K295R) forms of Src. Immunoprecipitation of Aurora-A-GFP revealed that Aurora-A and Src co-precipitated (Fig. 3a, lower panel). The inactive form of Src (K295R) was bound more efficiently to Aurora-A-GFP with respect to wt-Src or the Y527F-Src mutant (Fig. 3a, lower panel), thus suggesting that the inactive form of Src would be released more slowly from its substrate. Moreover, the possibility that Aurora-A is phosphorylated in living cells by Src was examined. The level of tyrosine phosphorylation of Aurora-A was higher in the lysates that overexpressed wt-Src and constitutively active Y527F-Src when compared with the cells that overexpressed the kinase-dead mutant K295R-Src (Fig. 3a, upper panel).

To further test this finding, HeLa cells were synchronized at the G1/S boundary and lysed 8 h after release from the S-phase (G2-enrichment). Some of the cells were transfected with Aurora-A-GFP and treated with PP2. The proteins phosphorylated at tyrosine were immunoprecipitated using an anti-phosphotyrosine antibody. As shown in Fig. 3b, Aurora-A was immunoprecipitated under control conditions, but was not immunoprecipitated in the presence of PP2, confirming that Aurora-A is phosphorylated at tyrosine(s) by Src.

In addition, immunoprecipitation of the endogenous proteins in synchronized HeLa cells, showed that endogenous Src and Aurora-A co-precipitated (Fig. 3c). To measure a possible modulation of that interaction quantitatively, we used the PLA as the method of choice to measure variations in the association between endogenous Aurora-A and Src. As confirmation of the cell cycle-dependent interaction between the endogenous proteins, the cells incubated with both anti-Aurora-A and anti-Src antibodies showed a low number of spots in S-phase (Fig. 3d—S-phase) and, as expected, a large number of red dot signals were detected in G2-phase, (Fig. 3d,e—ctl). The treatment with the Src inhibitor PP2 caused a strong reduction in the number of red dots28 (Fig. 3d,e—PP2), probably because PP2 binds to a deep pocket near the ATP-binding cleft of the Src family kinases29, thus causing a steric hindrance that can limit the interaction between Src and Aurora-A. Strikingly, a block of the Golgi-unlinking in G2-phase using JNK inhibitor VIII prevented the association between Aurora-A and Src, in line with our proposal that Src activation signals Golgi unlinking to Aurora-A (Fig. 3d,e—JNKI-VIII). Moreover, in cells blocked in S-phase and transfected with Aurora-A-GFP, as the endogenous Aurora-A protein content is undetectable in this phase, the fragmentation of the Golgi complex with BFA strongly enhanced Src-Aurora-A interaction (Fig. 3f), and thus further confirming that Src-Aurora-A interaction is promoted by Golgi fragmentation.

**Src phosphorylates Aurora-A to induce centrosome maturation.** To assess the functional relevance of Src-Aurora-A interaction, in vitro kinase assays were performed using recombinant GST-Src and untagged Aurora-A, and the extent of Aurora-A phosphorylation was determined using an anti-phospho-tyrosine antibody.
**Figure 2 | The Golgi ribbon unlinking in G2 induces local activation of Src in HeLa cells.** Cells grown on coverslips and processed for immunofluorescence confocal microscopy after S-phase block release. (a) Representative images of cells at the indicated cell cycle phase, labelled with anti-pY416-Src (green; insets), GM130 (red) antibodies and Hoechst 33342 (blue) for the nuclei. Scale bar, 4 μm. (b) Quantification of cells as in a showing fluorescence intensity of pY416-Src (A.U.). For the S-phase block (T-block), cells were treated with DMSO soon after the thymidine-block release; for the other conditions, the cells were incubated with DMSO (∅), PP2 (10 μM) or JNKi-VIII (50 μM) for 2 h before fixing (8 h after the S-phase-block release). For GM130 depleted cells (GM130 KD), interference was carried out for 24 h according to the synchronization procedure. GM130 KD cells were treated with vehicle (∅) or JNKi-VIII before fixing. All the images analysed were acquired at maximal resolution under fixed-imaging conditions. Equal areas were used to select the Golgi regions and a non-Golgi region with a similar background. Quantification data are mean values (± s.d.) from two independent experiments, each carried out in duplicate. Two-tailed Student’s t-tests were applied to the data (n = 50; P value < 0.05). (c) HeLa cells arrested in S-phase using the thymidine block, and, then grown under starving conditions (0.1% foetal calf serum (FCS)) for 1 h to downregulate Src activation because of the growth factors contained in the medium. Cells were then treated with DMSO (∅) or with 200 ng ml⁻¹ BFA for 5, 10, 30, 60 and 120 min in combination with PP2. Representative images of cells grown on coverslips and processed for immunofluorescence to monitor the BFA-induced extent of Golgi fragmentation. The cells were labelled with anti-Gal-T antibody (green; Golgi complex) and Hoechst 33342 for nuclei (blue). Images were acquired at maximal resolution under fixed-imaging conditions. Scale bar, 5 μm. (d) After the treatments as in c, cells were lysed with a buffer containing protease and phosphatase inhibitors. Equal amounts of clarified cell lysates were loaded onto SDS–PAGE and immune-blotted with anti-pY416-Src and anti-Src antibodies. (e) Quantification of d expressed as pY416-Src/Src. Two-tailed Student’s t-tests were applied to the data (P value < 0.05). A.U. arbitrary unit; DMSO, dimethylsulphoxide.
Aurora-A was phosphorylated by the kinase Src also in vitro, and the modification was almost absent if the Src catalytic activity was impaired by the Src inhibitors SU6656 or PP2 (Fig. 4a).

Next, we analysed Aurora-A activation, using a two-step kinase assay with Src and Aurora-A in the first step, and by subsequent incubation of the recombinant proteins with Histone-H3.

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**Figure 3 | Src physically associates to Aurora-A and this interaction is modulated by inhibition of Golgi fragmentation.**

(a) HeLa cells arrested in S-phase with double-thymidine block and transfected for 24 h with Aurora-A-GFP together with wt, constitutively active (Y527F) or kinase dead (K295R) Src mutants after the first release from thymidine. Immunoprecipitation was performed from G2-enriched population by incubating 1mg of clarified cell lysates with anti-GFP antibody, O/N at 4°C. Co-precipitated proteins were separated onto 8% SDS-PAGE gel and the extent of tyrosine phosphorylation was analysed by western blotting. (b) The phosphorylation on Aurora-A was examined also by immunoprecipitation with anti-phospho-tyrosine agarose-conjugate antibody after 24 h of overexpression of Aurora-A-GFP. Where indicated, 10μM PP2 was used for 2 h before protein extraction to assess the specificity of the Src-mediated phosphorylation. (c) HeLa cells synchronized with double-thymidine block, and lysed 10 h after the second thymidine washout. Five hundred microgram of total cells lysates were incubated with different amounts of anti-Src antibody (0.5, 1.5 or 3μg of total proteins) or with anti-rabbit pre-immune IgGs (3μg of total proteins), O/N at 4°C. After the incubation, the immune complexes were precipitated using protein A-Sepharose and SDS-PAGE was performed. The co-precipitated proteins were revealed with anti-Src and anti-Aurora-A antibodies. (d,e) HeLa cells grown on coverslips and arrested in S-phase with double-thymidine block. Two hours before fixing, cells were incubated with 10μM PP2 or 50μM JNKi-VIII. After 8 h without thymidine, the cells were processed under confocal microscopy with 2μgml⁻¹ Hoechst 33342 for visualization of nuclei (blue), and subjected to PLA with anti-Aurora-A and anti-Src antibodies. The interactions were expressed as the numbers of red dots each of which indicates the proximity of Src and Aurora-A. All images analysed were acquired at maximal resolution under fixed-imaging conditions. (d) Representative images of PLA as above described. Scale bar, 5μm. (e) The quantification of cells treated as in d. Data are means (± s.d.) from three different experiments. Two-tailed Student’s t-tests were applied to the data (n = 50; P < 0.001). (f) Representative images of PLA between endogenous Src and overexpressed Aurora-A-GFP was performed on synchronized HeLa cells treated with 200ng ml⁻¹ BFA for 2 h (+ BFA). Scale bar, 5μm.
a well-validated in-vitro Aurora-A substrate that is phosphorylated at Ser10 (ref. 30). Aurora-A-mediated phosphorylation of Histone-H3 on Ser10 was markedly increased when Src was present in the reaction, and was reduced when the catalytic activity of Src was inhibited by PP2 (Fig. 4b). In addition, we did not observe any enhancement of Aurora-A auto-activatory phosphorylation using the antibody that recognizes phosphorylated Thr288, suggesting that the catalytic activity of Aurora-A can be modulated even in the absence of phosphorylation at Thr288 (refs 31,32).

To identify the phosphorylated tyrosine residue(s) on Aurora-A, we performed a phosphoproteomic analysis of the recombinant Aurora-A incubated with Src, which showed that only Tyr148 is phosphorylated under our experimental conditions (Fig. 5a). This residue, which is evolutionarily conserved from yeast to mammals (Fig. 5b), has been found to be phosphorylated by a previous phosphoproteomic study33, but its physiological role has never been assessed. To determine whether Tyr148 of Aurora-A is modified also in vivo, we produced an Aurora-A mutant containing Tyr148 mutated into phenylalanine (Y148F) and performed immunoprecipitation experiments to analyse the extent of phosphorylation. HeLa cells were synchronized by the double-thymidine treatment and co-transfected with vectors for the expression of wt-Aurora-A-GFP or the phospho-depleted Aurora-A mutant (Aurora-A-Y148F-GFP), together with an untagged GFP, the phospho-depleted (Y148F) and the phospho-mimetic (Y148D) Aurora-A mutants (Fig. 5c). As shown in Fig. 5c,d, the phosphorylation of wt-Aurora-A was reduced in the presence of Src inhibitor PP2. An analogous-reduced phosphorylation degree was observed for the Y148F-Aurora-A phospho-depleted mutant, which showed a background level of phosphorylation that was comparable to that of the wt protein where the Src catalytic activity was blocked by PP2 (Fig. 5c,d).

Next, to examine the functional role of this phosphorylation, G2-enriched HeLa cells were transfected with wt-Aurora-A-GFP, the phospho-depleted (Y148F) and the phospho-mimetic GFP-mutants (Y148D). The GFP-tagged proteins were immunoprecipitated and tested in an in vitro kinase assay. As expected, the phospho-depleted mutant protein was less active compared with the wt counterpart (Supplementary Fig. 4a). In addition, also the phospho-mimetic mutant was inactive (Supplementary Fig. 4a). This is not surprising because while phenylalanine can well mimic the structure of a non-phosphorylatable tyrosine, aspartate, being much shorter than phosphotyrosine, and lacking the aromatic ring, should not properly mimic tyrosine replacement, thus not behaving as a phosphorylatable residue34. Importantly, as a further evidence of the functional role of this phosphorylation, the phospho-depleted mutant of Aurora-A showed a reduced association with the centrosome, which was comparable with the reduced centrosomal recruitment caused by Src inhibition with PP2 (Fig. 6a,b), and in line with the results showed in Fig. 1a. The residual centrosomal recruitment of Aurora-A-Y148F-GFP mutant is likely related to a Src-independent event. In line with the lack of catalytic activity, also Aurora-A-Y148D-GFP fails to be recruited to the centrosome (Supplementary Fig. 4b).

Next, we focused on centrosome maturation, which is one of the key Aurora-A-dependent preparatory events for mitotic ingress and proper chromosomal segregation. Although the centrosomal maturation occurs via a still debated mechanism, it is the result of the recruitment of long coiled-coiled centrosomal proteins, such as Cep192, pericentrin (PCNT), and Cep215 (also called Cdk5-Rap2)35, which form a centrosomal matrix that acts as a scaffold for the recruitment and activation of the mitotic kinases and for the recruitment of microtubules (MT) nucleating and organizing factors36. Among them, the most critical is the γ-tubulin ring complex (γ-TuRC), which serves as a MT-nucleating pole. A timely and ordered centrosomal maturation is pivotal for the formation of the spindle and for chromosome segregation. Any imbalance of this process is at the basis of the chromosomal instability.

Given that Aurora-A phospho-depleted mutant (Y148F) showed a reduced centrosomal localization (Fig. 6a,b), we tested whether the overall process of centrosomal maturation, of which Aurora-A is part of37, was compromised, which could then affect the entry into prophase. Synchronized HeLa cells were depleted of endogenous Aurora-A and transfected for 9 h with siRNA-resistant constructs (Supplementary Fig. 5a) for the expression of wt-Aurora-A-GFP and the phospho-depleted GFP-mutant (Y148F). In addition, to compare the relevance of the newly identified phosphorylated site (Tyr148) with the well-described Thr288, we generated and tested a siRNA-resistant phospho-depleted T288A-GFP and a double siRNA-resistant Aurora-A-Y148F/T288A-GFP mutant. The cells were fixed when they were mostly in G2 and examined by confocal microscopy for quantitative measure of the levels of centrosome-
Figure 5 | The mutant Aurora-A-Y148F showed an impaired phosphorylation. (a) Phosphorylation of Aurora-A at Tyr148 as determined by mass spectrometry analysis. CID fragmentation spectra of two modified peptides bearing the phosphorylation at Tyr148. (upper) MS/MS of the triply charged phosphopeptide (142–151) GKFGLPYPYLR (m/z 402.76, MH⁺ equal to 1206.27). (lower) MS/MS of the doubly charged phosphopeptide (144–151) FGNYVYLR (m/z 510.74, MH⁺ equal to 1020.46). (b) Alignment of the sequences surrounding human Aurora-A Tyr148 from various species. Y148 site is shown in red. (c) HeLa cells were arrested in S-phase using the double-thymidine block. After the first release from thymidine, cells were transfected for 24h with wt-Aurora-A-GFP or Aurora-A-Y148F-GFP in combination with wt-Src. 2h before lysis, cells were treated with 10 μM PP2 where indicated. Immunoprecipitation was performed by incubating 1mg of clarified cell lysates using an anti-GFP antibody, O/N at 4°C. The precipitated proteins were separated onto 8% SDS–PAGE gels and the extent of tyrosine phosphorylation was analysed by western blotting using an anti-phospho-tyrosine antibody. Representative western blotting of two different experiments. (d) Quantification of c expressed as pY/Aurora-A. Data are means (± s.e.m.) of two different experiments.

Discussion

Precise spatio-temporal localization and activation of mitotic kinases is crucial for the accurate sequential organization of mitotic events. In this study, we have built upon our previous findings that showed that Golgi ribbon unlinking is required for the centrosomal recruitment of the mitotic kinase Aurora-A, which is a key regulator of G2/M transition, centrosome maturation and spindle formation.

Here we have revealed a key element of the signalling pathway that connects Golgi fragmentation to Aurora-A recruitment. Co-immunoprecipitation experiments and PLA showed that a member of the Src family kinases interacts with Aurora-A during G2/M, and that this interaction depends of the state of dis(assembly) of the Golgi ribbon. We show that Src phosphorylates Aurora-A at Tyr148, and that this phosphorylation increases the kinase activity of Aurora-A. Finally, we find that this phosphorylation is crucial for centrosomal maturation. Centrosome maturation involves the recruitment of pericentriolar material components (PCM), such as Cep192/SPD-2, PCNT/PLP, and Cep215 (also called Cdk5Rap2) to centrosomes and to regulate its maturation, which in turn, is a prerequisite for orderly spindle formation and for chromosome segregation.
data is that the reduced level of Aurora-A at the centrosome, which is seen after a block of Golgi fragmentation, is not indicative of reduced affinity for a scaffold but is instead the direct consequence of reduced growth of PCM proteins as reported in the model proposed in Fig. 7.

Aurora-A is a proto-oncogene that is overexpressed in several human cancers and is required at several stages as cells progress towards and through mitosis. Aurora-A levels increase in G2-phase, when the protein is targeted to the centrosome through mechanisms that are only partially understood. Most probably, different scaffolds regulate the recruitment to the centrosome for different specific functions where, according to the classical view, Aurora-A is activated upon phosphorylation at Thr288. Aurora-A activity is protected by the action of phosphatases through the binding of Aurora-A itself to scaffold proteins like HEF1 and Ajuba, which relocate from the focal adhesions to the centrosome during G2/M transition, thus revealing a ‘point of dialogue’ between focal adhesions and the

Figure 6 | The unphosphorylatable Aurora-A at Y148 fails to recruit to the centrosome and impairs centrosomal maturation. (a) Synchronized HeLa cells were transfected for 24 h with wt-Aurora-A or Aurora-A-Y148F tagged with a GFP reporter. Where indicated, cells overexpressing wt-Aurora-A were also treated with 10 μM PP2 for 2 h before fixing. Cells were processed for immunofluorescence to measure the fluorescence intensity of the recruited Aurora-A-GFP, and an anti-γ-tubulin antibody was used as reference for the centrosome (A.U.). All images for the quantification were acquired at maximal resolution, under fixed-imaging conditions. Equal circle areas were used to select the centrosomes regions and a non-centrosome region with a similar background. Quantification data are means (± s.d.) from three independent experiments. Two-tailed Student’s t-tests were applied to the data (P<0.05). (b) Representative images of the cells quantified in a. Scale bar, 2 μm. (c,d) Synchronized HeLa cells were co-transfected for a short time period (9 h) with Aurora-A siRNA and siRNA-resistant wt Aurora-A-GFP, Aurora-A-Y148F-GFP, Aurora-A-T288A-GFP or a vector carrying both the mutations (Y148F/T288A). GFP-positive cells were analysed to measure the fluorescence intensity of the recruited PCNT (c) or γ-tubulin (d) to the centrosome. The images acquisition and the quantifications were performed as described in a. Quantification data are the mean values (± s.d.) from three independent experiments. Two-tailed Student’s t-tests were applied to the data (for PCNT P<0.0001; for γ-tubulin P value = 0.0002). Scale bars, 5 μM. (e,f) Synchronized HeLa cells were incubated for 2 h with 10 μM of the Src inhibitor PP2 and then processed for immunofluorescence analysis as described in a. A.U., arbitrary units.
Figure 7 | Aurora-A is an integrator of p-Tyr and p-Thr/Ser-based signalling to regulate centrosome maturation and mitotic entry. (a) Cleavage of the Golgi ribbon into stacks during the G2-phase of cell cycle induces the activation of a Golgi-based pool of SFK, and, subsequently, the interaction of SFK with Aurora-A. (b) Golgi-activated SFK, then phosphorylates Aurora A (Y –); this phosphorylation results in increased Aurora-A kinase activity and centrosomal recruitment. Whether Aurora is phosphorylated at the centrosome or in the cytoplasm by SFK, is not known. The additional phosphorylation of T288 in the activation loop (– T) by either trans- or auto-phosphorylations triggers a complex signalling cascade (c) that allows the recruitment of additional Aurora-A, PCTN and γ-tubulin for full centrosome maturation and G2/M transition. Aur-A, Aurora-A; CE, centrosome.

Through its ability to serve as a scaffold for anchoring several centrosomal proteins, PCNT is involved in key centrosome functions, including G1/S and G2/M-transitions, centrosomal maturation and mitotic spindle organization/orientation. For example, recent studies demonstrated that the absence of PCNT at the centrosome is associated with microcephalic osteodysplastic primordial dwarfism type-II (MOPD-II), a human autosomal recessive genetic disorder. Thus, our findings open the possibility that the genetic disorder MOPD-II can also rely on Aurora-A mutations. Further in vitro functional investigations and genetic studies on patients cohorts are needed to address this question.

The next important questions are to identify the precise member of the Src family kinases involved in Aurora-A phosphorylation and how the cleavage of the Golgi ribbon is sensed and signalled to Src. Key players in keeping the ribbon structure are the Golgi stacking factors GRASP55 and GRASP65. They have a pro-ribbon function that is reverted by specific phosphorylations. GRASP55 is phosphorylated by the RAF/MEK/ERK kinase module; GRASP65 is phosphorylated in Ser277 by Jnk2 (ref. 19) and by Plk1 in Ser189 (ref. 44). Conversely, the fission-inducing protein BARS is actively involved in the cleavage of the membranous tubules connecting the Golgi stacks. Src is a non-receptor tyrosine kinase with known roles in the transduction of signalling cascades that arise from receptors at the plasma membrane. It has also been shown that Src co-localizes with Golgi markers and is present in Golgi-enriched membrane fractions, which suggested an involvement of Src in the regulation of some of the many Golgi functions. Indeed, Src is activated at the Golgi apparatus by the arrival of cargoes from the ER in a mechanism that involves Gq (ref. 47) and is required for both intra-Golgi and post-Golgi trafficking. This event appears to be not related to Src-activation upon Golgi fragmentation as it is confined to a Golgi compartment different from the TGN. Src can also control post-Golgi traffic through phosphorylation of...
dynamin2, which is required for Golgi to plasma membrane delivery of cargo. It is unlikely that Src modulates Aurora-A as a consequence of the regulation of post-Golgi trafficking because we excluded any active involvement of Src in the cleavage of the ribbon and we assessed that Src activation becomes independent of JNK when the Golgi complex is forced to be in the form of mini-stacks, which are traffic competent.

Most relevant to the present study, Src has been shown to be involved in G2/M transition of the cell cycle in fibroblasts and more generally, there is a plethora of indications that supports a key role for Src in the regulation of the cell cycle. As suggested by the present study, and in line with literature data, the localisation of Src in different subcellular cytoplasmic compartments provides a means of spatially controlling these strong and promiscuous protein kinases. This in turn would guarantee that activation occurs only at the correct location and/or in the correct compartment, thereby conferring functional specificity and ensuring cell viability.

As mentioned for Aurora-A, also Src has a well-established role in tumorigenesis, supporting tumour growth, invasion and metastasis. It has been reported that, in cancer cell models, specificity and ensuring cell viability. and/or in the correct compartment, thereby conferring functional guarantees that activation occurs only at the correct location and compartments provides a means of spatially controlling these strong and promiscuous protein kinases. This in turn would guarantee that activation occurs only at the correct location and/or in the correct compartment, thereby conferring functional specificity and ensuring cell viability.

In conclusion, the data presented herein identify the kinase Src as a downstream component of the molecular machinery that is activated upon Golgi ribbon breakup, at the onset of mitosis. This specific and compartmentalized Src activation regulates the process of centrosome maturation through phosphorylation at Tyr148 and, probably, in coordination with phosphorylation of T288, thus promoting the entry into mitosis and the proper chromosome segregation.

**Methods**

**Cell culture and cell cycle synchronization.** NRK and HeLa cells were from the American Tissue Type Collection (ATCC, USA) and were grown in DMEM and MEM, respectively ( Gibco), supplemented with 10% fetal calf serum (Bioxchim), 1% MEM non-essential amino-acid solution, 1% L-glutamine, 1 U ml−1 penicillin and 50 μg ml−1 streptomycin (all Invitrogen). Cells were grown under a controlled atmosphere in the presence of 5% CO2 at 37 °C. For the cell cycle synchronization of HeLa cells, the population was treated with 2 mM thymidine; after incubation with thymidine, cells were washed and grown in complete medium of HeLa cells, the population was treated twice O/N with 2 mM thymidine; after each incubation with thymidine, cells were washed and grown in complete medium O/D. For the cell cycle synchronization of NRK cells, the population was treated with aphidicolin O/N and the day after cells were released from the aphidicolin block. The G2/M phase of NRK and HeLa cells was induced after 8 h from the washout.

**Flow cytometry.** Trypsinized HeLa cells were pelleted, washed in cold PBS, resuspended in ice-cold ethanol while vortexing. The cells were incubated O/N at 4 °C. The next day, the ethanol was removed by centrifugation and the cells were washed in cold PBS and incubated with 50 μg ml−1 propidium iodide (Invitrogen) for 30 min in the presence of RNase (Sigma). The cells were then analysed using Becton Dickinson FACSCantoA instrument (BD). The data were plotted with Diva software, with 20,000 events analysed for each sample.

**Cell transfection and RNA interference.** HeLa cells were transfected with the TransIT-LT1 Transfection Reagent (Mirus), according to the manufacturer’s instructions. The cDNA of full-length Aurora-A fusion protein and a gift from Dr Stefano Ferrari (Institute of Molecular Cancer Research, University of Zurich, Zurich, Switzerland). The cDNA of wt-Src, Y527F-Src and K295R-Src were a generous gift from Dr Gabriella Castoria (Seconda Università Degli Studi di Napoli—SUN, Italy). The phospho-mimetic Y148D, the siRNA-resistant wt-Aurora-A, phospho-depleted T288F, phospho-depleted Aurora-A T288A and the double Aurora-A Y148F/T288A mutants were obtained through mutagenesis of Aurora-A-GFP using QuickChange Site-Directed mutagenesis kits (Agilent). The primers used in the mutagenesis reactions are shown in Supplementary Table 1.

After transfection, the intracellular protein contents were analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by western blotting, and the cells were further processed according to the experimental design. siRNA duplexes were transfected using Lipofectamine 2,000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer instructions. GM130 mRNA was targeted using siRNA duplexes directed against the sequence 5′-AGTTTAGATGACGGAACTGTC-3′ (Dharmacon; Lafayette, CA, USA). Non-targeting siRNA sequences were used as controls. Aurora-A siRNA and siRNA-resistant constructs were co-transfected using jetPRIME (Polyplus transfection, Illkirch, France), according to the manufacturer instructions. Aurora-A mRNA was targeted using siRNA duplexes directed against the sequence 5′-TCCTGAGCTGTAGTGATTTA-3′ (Dharmacon; Lafayette, CA, USA).

**Antibodies and reagents.** Thymidine, BFA and fibronectin were from Sigma-Aldrich. Dimethylsulphoxide was from Carlo Erba (Italy). JNK-VIII, PP2, p38α and p38β antibodies were from Calbiochem. U0126 and Mowiol were from (USA). SU6656 was from Millipore (USA). Hoechst 33342, Alexa Fluor-conjugate secondary antibodies (1:400, A21201; A10037; A21206; A10442) and anti-goglon97 (A21270; 1:100) were from Molecular Probes (OR, USA). The primary antibodies were from the following sources: anti-phospho-histone H3 (pSer10) for western blotting (1:1,000) or for immunofluorescence (1:100; 6-570), anti-phospho-tyrosine (clone 4G10) (1:1,000; 05-321), anti-phospho-tyrosine Platinum agarose-conjugate (16–638) from Millipore (Billerica, MA, USA); anti-Histone H3 (1:1,000; sc-10809), anti-mouse (sc-2025) and anti-rabbit (sc-2027) pre-immune IgGs (1:5,000) from Santa Cruz; anti-GAPDH (1:8,000; 4,699-5,535) from AbD Serotech (USA); anti-Aurora-A (1:1,000; 610,938), and anti-GM130 (1.50; 610,823) from BD Biosciences (San Jose, CA, USA); anti-Src (clone 36D10, 1:1,000; 2,109), anti-Src-pY416 (clone D949G, 1:1,000; 6,943) for western blotting, anti-Aurora-A (1:1,000; 3,092), anti-Aurora-pT288 (1:500; 3,079) from Cell Signalling; anti-Src-pY416 (1:100; 44-660A1) for immunofluorescence from Invitrogen; anti-Gal-T (1:250; HPA006729) and anti-α-tubulin (1:200; T3539) from Sigma-Aldrich; anti-GFP (1.5μg per mg, clone 9F6; F9182, 1:1000; Ab2188), anti-GFP (1:1,000: Ab299), anti-TGN46 (1:250; Ab50995), anti-giactin (1:500; Ab93281) and anti-PCNT (1:500; Ab28144) from Abcam; anti-α-Mannosidase II (1:150; HPA040627) from Atlas Antibodies (Stockholm, Sweden).

**Kinase assay.** Aurora-A was expressed as intein—Aurora-A fusion protein in the BL21 (DE3) Escherichia coli strain. The soluble fraction from bacterial extracts was loaded on to a 10 ml chitin column (New England Biolabs). The bound fraction was washed with 100 mM DTT (dithiothreitol) to induce in vitro self-splicing. A non-radioactive reaction was performed using 500 μg purified Aurora-A incubated with 50 ng Src (Enzo Life Sciences) in 50 μl kinase buffer (100 mM Tris-HCl, pH 7.2, 3 mM MgCl2, 1 mM NaF, 1 mM DTT) for 1 h at 37 °C in the presence of 1 mM ATP (Amersham Biosciences). Then, 1 μg recombinant His-tagged histone-H3 (Enzo Life Sciences) was added to the kinase reaction mix for 15 min at 37 °C. For the pre-incubation, 1 μM PP2 was added for 30 min at 37 °C, before the addition of Aurora-A. Then the samples were analysed by SDS–PAGE.

**Immunoprecipitation.** For each immunoprecipitation, HeLa cells were lysed after reaching 80% confluency, using 500 μl ice-cold extraction buffer containing 20 mM HEPES, pH 7.5, 10 mM NaCl, 15 mM MgCl2, 10 mM NaF, 1 mM EDTA, 1 mM Na2VO4, 0.5% (w/v) Nonidet-P-40, supplemented with protease and phosphatase inhibitors cocktail (Roche). After 30 min of cell lysis on ice in the extraction buffer, the samples were centrifuged at 6,000 g for 30 min at 4 °C. The supernatants were then normalized to 500 μg and incubated under rotation at 4 °C with 1.3 μg anti-GFP antibody, as indicated for 2 h or O/N, before addition of protein A/G-Sepharose for a further 30 min. When using the anti-phospho-tyrosine antibody conjugated to the agarose matrix (4G10, Platinum, Millipore), the resin was then washed extensively in extraction buffer and then 20 μl of recombinant resin was used for each sample, incubating O/N on a rotating shaker. The beads were washed twice with 500 μl ice-cold complete extraction buffer, and twice with 500 μl ice-cold extraction buffer without detergent. The western blots were visualized using the ECL reagents (GE Healthcare), according to manufacturer instructions. Cells for ECL-based detection, Where stated, band intensities were quantified using Quantity One software (Bio-Rad Laboratories).

Full-scan images of all western blotting data are reported in Supplementary Fig. 6.
Golgi fragmentation assay in permeabilised NRK cells

scanning confocal microscopy.

manufacturer's instructions. The amplified signals were analysed using laser
in situ
avoid 'pixel saturation'. For the experiments in which
of the centrosome in the mentioned experiments (see Fig. 6 and Supplementary

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G2-arrest, or for 6 h with 10
block were either fixed in S-phase, or treated for 18 h with bis-benzimide, for
quantitative analysis of the phenotype of the Golgi
function. All of the images were acquired at maximal resolution, under
count the above-threshold fluorescent 'Golgi objects', using the 'Analyse Particle'

Proximity ligation assay.

Golgi object' assays. Hela cells arrested in S-phase using the dual-thymidine block were either fixed in S-phase, or treated for 18 h with bis-benzimide, for G2-arrest, or for 6 h with 10 μM U0126, or for 2 h with 10 μM SU6656. For quantitative analysis of the Golgi integrity (number of objects per Golgi), the Golgi-association fluorescence of randomly chosen cells was acquired. The freeware ImageJ software package was used to automatically apply a threshold and to count the above-threshold fluorescent 'Golgi objects', using the 'Analyze Particle' function. All of the images were acquired at maximal resolution, under fixed-imaging conditions. For qualitative analysis of the phenotype of the Golgi apparatus, the GM130 staining was processed with the freeware ImageJ software package using the ‘Invert’ function.

Golgi fragmentation assay in permeabilised NRK cells. NRK cells were grown to 60% confluency on fibronectin-coated glass coverslips and incubated with 2 mM thymidine for 10–12 h to arrest cells in S-phase. Then, the cells were washed and incubated O/N with 500 ng ml
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Phosphoproteomic analysis

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Author contributions
M.L.B. designed and performed all the experiments, analysed the data and wrote the manuscript. D.S. performed experiments, analysed the data and contributed to the writing of the manuscript. C.D.A. performed the phosphoproteomic study and analysed the data. R.C. performed initial experiments. A.S. supervised the phosphoproteomic study and contributed to the critical revision of the manuscript. D.C. contributed to the supervision of the study and to the critical revision of the manuscript. A.C. conceived and supervised the whole study and contributed to the writing of the manuscript.

Additional information
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