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Uncovering new substrates for Aurora A kinase

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Aurora A is a serine/threonine kinase that is essential for a wide variety of cell-cycle-related events, but only a small number of its substrates are known. We present and validate a strategy by which to identify Aurora A substrates and their phosphorylation sites. We developed a computational approach integrating various types of biological information to generate a list of 90 potential Aurora substrates, with a prediction accuracy of about 80%. We also demonstrated the specific phosphorylation of NUSAP (nucleolar and spindle-associated protein) by Aurora A *in vivo*. Our results provide a means by which to develop an understanding of Aurora A function and suggest unexpected roles for this kinase.

Keywords: Aurora substrates; pattern discovery; phosphorylation motifs

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INTRODUCTION

The aurora kinases are a family of closely related serine/threonine protein kinases, with three members in metazoans (A, B and C) that are functionally related to different cancers (Mahadevan & Beeck, 2007). In humans, the gene which codes for Aurora A (AurA) is often amplified in primary tumours and cancer cell lines (Bischoff *et al*, 1998), and AurA and AurB overexpression is associated with poor prognosis in several tumour types (Naruganahalli *et al*, 2006). The Aurora kinases are important in cell division and the maintenance of genome stability. The functions of each family member are linked to their specific localization at critical cell-cycle times (Vader & Lens, 2008). AurA is a centrosomal kinase that participates in cell-cycle progression and has a well-characterized role in centrosome maturation during late G2 and prophase, as well as in spindle assembly (Sardon *et al*, 2008). AurB localizes to the nucleus in interphase, to the kinetochores and the spindle midzone in anaphase and is required for chromosome bi-orientation and cytokinesis (Vader & Lens, 2008). AurC acts during meiosis, and its function is closely related to that of AurB in cytokinesis (Slattery *et al*, 2009). The three Aurora kinases have a similar protein domain organization, with divergent amino-terminal and carboxy-terminal domains and a conserved catalytic domain. AurA and AurB show specificity for certain substrates, but also share some of them *in vitro*, including HH3 and MBP. A consensus motif for these kinases was first determined for Ipl1, the only Aurora kinase in the budding yeast *Saccharomyces cerevisiae* (Cheeseman *et al*, 2002).

Although the participation of Aurora kinases in the cell cycle is well established, their mechanism of action is poorly understood and the relatively small number of their substrates that are known do not account for their many functions. Thus, the identification of Aurora substrates is important for a global understanding of their functions.

Here, we present and validate a new strategy for identifying new substrates for AurA kinase. By analysing the available data on AurA substrates and their phosphorylation sites, we developed a computational approach that uses distinct types of biological information to generate a ranked list of potential Aurora substrates. We then validated our predictions in experiments with a group of candidates by using *in vitro* kinase assays and mass spectrometry analyses. Finally, we also demonstrated the specific phosphorylation of NUSAP (nucleolar and spindle-associated protein) by AurA *in vivo*.

RESULTS AND DISCUSSION

Identification of potential substrates for AurA

The consensus motif of the Aurora kinase was first characterized in *S. cerevisiae* (Cheeseman *et al*, 2002) as [KR].[ST][ILV]. Two different patterns have been proposed for the human AurA kinase: [KNR]R.[ST][AFILMV] (Ferrari *et al*, 2005) and R.[ST][ILV] (Ohashi *et al*, 2006), the latter being a more restricted form of the yeast motif. However, these motifs only match 8, 5 and 7, respectively, of the 19 known phosphorylation sites of AurA. To broaden the coverage, we aligned the site-containing regions from the list of known AurA phosphorylation sites and defined a more lenient motif—[KR].[ST][^P]—in the light of a report by Ferrari *et al* (2005) showing that proline at position +1 abolishes phosphorylation by AurA. This 'notP motif' matches 13 of the 19 sites. It also matches most of the known substrates of AurB (15 of 18

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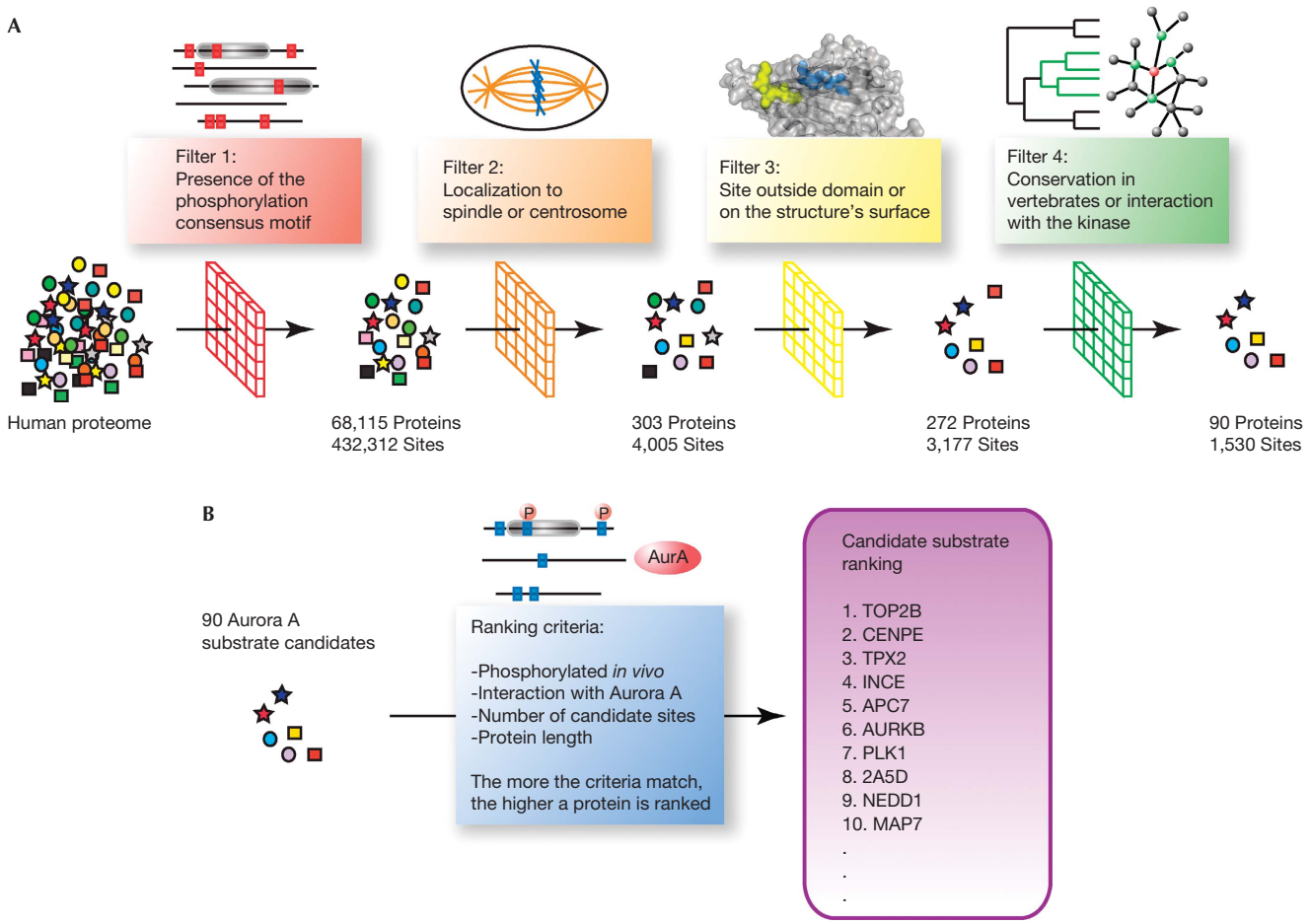

Fig 1 | Schematic representation of the bioinformatics approach. (**A**) Candidate substrate selection. Aurora substrate candidates were selected on the basis of a series of filters applied to the whole human proteome: presence of an Aurora phosphorylation motif in the sequence, localization to the centrosome or the spindle, accessibility of the consensus motif and conservation of the potential phosphorylation site among vertebrates. (**B**) Ranking of candidate Aurora substrates (see main text for details).

known AurB phosphorylation sites). This is not surprising, as the motif is an extension of the yeast pattern and Ipl1—the only yeast Aurora kinase—shares functions and substrates with both AurA and AurB. In fact, recent data suggest that in vertebrates, the substrate specificity of these two kinases is primarily a consequence of their different subcellular locations and/or interactions with specific cofactors (Fu *et al*, 2009; Hans *et al*, 2009).

The notP motif matched 432,312 sites in 68,115 of 77,683 non-redundant protein sequences in the human proteome, many of which are probably false positives. We therefore developed a filtering strategy with which to increase the specificity of our predictions by integrating different types of contextual data including subcellular localization, *in vivo* phosphorylation, interactions with the kinase, site accessibility and motif conservation across vertebrate species (Fig 1).

As AurA specifically localizes to the centrosomes, spindle poles and spindle microtubules (Carmena & Earnshaw, 2003), we focused our analysis on the subset of proteins associated with these structures, as identified by large-scale proteomic analyses of human cells (Andersen *et al*, 2003; Sauer *et al*, 2005; Nousiainen *et al*, 2006). We also included proteins that were annotated as localizing to the spindle in the GeneOntology database (Ashburner *et al*, 2000; Fig 1), some of which localize to the kinetochores and could therefore be substrates of AurB. By reducing the number of potential candidates from 77,683 human proteins to 308, we restricted our analysis to the specific biological context of the Aurora kinases, thereby reducing the possibility of false-positives, but also risking discarding some substrates.

As phosphorylation motifs need to be accessible to their corresponding kinase, they are often located in hinge and loop regions outside globular domains (Neduva *et al*, 2005; Gnad *et al*, 2007). We therefore used information from high-resolution three-dimensional structures—when available—to calculate the accessibility of each site and retained only those on the protein surface. If no reliable structural information was available we excluded the sites inside domains, although this means that we potentially discarded a few real ones. Phosphorylation sites that were predicted to be outside globular domains were always considered accessible (Fig 1).

Functionally relevant phosphorylation motifs are expected to be better conserved during evolution than those that appear randomly (Malik *et al*, 2008). We therefore included a further

filtering step, to eliminate biologically irrelevant hits. To assess the conservation of candidate sites, we created sets of orthologues for all proteins in our spindle/centrosome set from the Ensembl database (Flicek *et al*, 2008). We limited the conservation analysis to include only vertebrates, because short motifs usually evolve quickly and can therefore only be detected in closely related species (Neduva *et al*, 2005). As phosphorylation sites often occur outside domains (Gnad *et al*, 2007) and are fast evolving (Neduva *et al*, 2005), typical multiple sequence alignment strategies would not provide sensible results (Chica *et al*, 2008; Perrodou *et al*, 2008). We therefore devised two alternative ways of assessing motif conservation.

Our first strategy was to examine whether the motif was found at a similar position in the human sequence and the corresponding orthologue, allowing a deviation of 1% in the position. As the relative positions of sites might not be preserved across domain insertions and deletions or gene fusion events, this method could only be applied to orthologues of similar length.

Our second strategy used the protein Basic Local Alignment Search Tool (BLAST; Altschul *et al*, 1997) to search for locally matching sequence stretches in orthologues containing the phosphorylation motif. As protein BLAST produces local alignments of the residues surrounding the given phosphorylation sites, this approach is also applicable in cases of non-uniform changes in sequence length in some species, and thus did not require any restriction for selecting the set of orthologues. Similar approaches have been used by Malik *et al* (2008) to identify functionally relevant phosphorylation sites.

We only considered sites in the top five ranks for each substrate candidate that were conserved in at least 90% of the orthologues by one of our methods. Among the known AurA substrates, 12 of 19 phosphorylation sites were ranked in the top five according to the notP motif conservation. The complete filtering procedure reduced the number of candidate substrates to 90, with 347 potential phosphorylation sites conserved (Fig 1).

Finally, we ranked the candidates by using a scheme that awards one point to proteins (i) interacting directly or indirectly with AurA, (ii) with conserved potential phosphorylation sites according to the presence-based method or (iii) with conserved potential phosphorylation sites according to the BLAST-based method. Two extra points were attributed to proteins with a notP motif coinciding with a known *in vivo* phosphorylated site (Nousiainen *et al*, 2006; Dephoure *et al*, 2008), which strongly indicates it is a real substrate. In substrate candidates with the same number of points, we considered the number of sites phosphorylated *in vivo* and conserved according to both the presence- and BLAST-based method ('threefold overlap'), as well as the number of sites fulfilling at least two of these three criteria ('twofold overlap'). The final ranked list of candidate substrates for Aurora kinases and their predicted phosphorylation sites are shown in Fig 2.

Experimental validation of the predictions

To test the predictions of our analyses we performed AurA *in vitro* phosphorylation assays on a set of ten candidates selected from throughout the ranked list on the basis of the availability of full-length complementary DNA clones in appropriate vectors for expression in mammalian cells (Fig 2). Kinase assay conditions were set on the known AurA substrates TPX2 (Kufer *et al*, 2002) and TACC3 (Kinoshita *et al*, 2005), each with a different tag (TPX2–FLAG and GST–TACC3). As a negative control, we used DYNLL1—a protein that is present in the spindle/centrosome proteome but lacks the consensus motif for AurA phosphorylation (Fig 3A).

The kinase assays showed that 8 of the 10 candidate substrates incorporated ^{32}P on incubation with AurA (Fig 3B), whereas 2—YWHAG (14-3-3γ) and YWHAE (14-3-3ε)—did not, suggesting that they are not AurA substrates *in vitro* (Fig 3C). In order to quantify the results and compare ATP incorporation between substrates, we normalized the autoradiography band signals to exclude differences due to the time of film exposure and the amount of protein (supplementary Fig S1A online), concluding that CENT1, DYNC1LI1, TUBB4, TUBG1, MAP7, NUSAP and SPIN are phosphorylated by AurA *in vitro*. However, we found no correlation between the number of predicted phosphorylation sites of a protein and the amount of ATP that it incorporated, suggesting that not all the sites are phosphorylated and/or that they show different affinities for the kinase. As the level of anaphase-promoting complex 7 (APC7) phosphorylation was much lower than that for the other candidates, we confirmed phosphorylation at the predicted site Ser 85 in the AurA-treated sample by using mass spectrometry (supplementary Fig S1B,C online).

As the notP motif also matches the AurB recognition sites, we tested whether the selected substrates were also phosphorylated by AurB *in vitro*, with RacGAP1 as a positive control (Minoshima *et al*, 2003). We found that AurB weakly phosphorylated all confirmed AurA substrates (Fig 3B) and did not phosphorylate YWHAG and YWHAE (Fig 3C). However, AurA and AurB phosphorylation concentrations should not be directly compared, as it has been shown that AurB activity *in vitro* is lower than that of AurA (Eyers *et al*, 2005). Overall, our experimental validation assays showed that 8 of 10 candidate proteins are Aurora substrates *in vitro*—a prediction success rate of about 80%. The accuracy with respect to individual phosphorylation sites remains to be assessed.

To determine whether these candidates are AurA substrates *in vivo*, we immunoprecipitated recombinant NUSAP from transfected interphase cells or mitotic monastrol-arrested cells, with or without the selective AurA inhibitor MLN8237 (Fig 4). The mitotic or interphase state of the cells in the different conditions was confirmed by fluorescence-activated cell sorting analysis (supplementary Fig S2 online). Mass spectrometry analyses identified a phosphorylated peptide—containing the predicted site Ser 240—in the sample obtained from the mitotic cells (Fig 4C). This phosphopeptide was absent in interphase cells and strongly reduced in mitotic cells treated with the inhibitor (Fig 4A). To test whether AurB could also phosphorylate NUSAP, we performed an MS/MS analysis on recombinant NUSAP incubated with the AurB inhibitor ZM447439 (Fig 4D). We did not find any difference between the amounts of phosphorylated peptide observed in mitotic cells with or without inhibitor (Fig 4B), suggesting that AurB does not phosphorylate NUSAP Ser 240. These data suggest that NUSAP is an AurA substrate *in vivo*.

One of our other predictions—that CENP-E Thr 422 is phosphorylated by AurA and AurB—has been validated *in vivo* (Kim *et al*, 2010) since we performed our analyses, providing further support for our strategy.

Rank	Prot name	UniProt ID	Phosphorylation sites
1	TOP2B	Q02880	S50, S129, T1371, **S1550**, **S1576**
2	CENPE	Q02224	S201, **T422**, S434, **S454**, T626, S789, S1320, T1505, S1562, T1671, T1712, S2048, S2119, S2314, T2450, S2468
3	TPX2	Q9ULW0	**S121**, **S125**, **S359**, S729, S742
4	INCE	Q9NQS7	S72, S87, S91, S94, S452, **T897**, **S898**
5	APC7	Q9UJX3	S80, **S85**, S322
6	AURKB	Q96GD4	**T232**
7	PLK1	P53350	**T210**, T259
8	2A5D	Q14738	**S88**, S454, T550, **S573**
9	NEDD1	Q8NHV4	S171, S176, S293, **S423**, S637
10	MAP7	Q14244	T146, S161, S181, **S200**, S705
11	KIF11	P52732	T222, T223, T370, **S931**
12	CENPC	Q03188	T183, **S261**, **S763**
13	Q8IW27	Q8IW27	T183, **S261**, **S763**
14	KIF4A	O95239	**T799**, **S1028**, **S1126**
15	CND1	Q15021	S442, T454, **S585**, T761, T1203, **T1388**, S1395
16	CE170	Q5SW79	T116, S126, **S312**, S472, **T501**, **T628**, S829, S952, **S958**, S968, S980, T1074, T1090, T1098, S1101, S1123, T1156, S1210, T1405
17	KIF1B	O60333	T1122, T1295, S1347, T1439, **S1658**
18	SMC1A	Q14683	**S970**
19	TOP2A	P11388	**S1387**
20	RGAP1	Q9H0H5	**S144**, T472, **S573**, **T601**
21	HASP	Q8TF76	**S93**, **S143**, T660
22	RBP2	P49792	S352, T714, S778, S786, S1912, T2192, T2398, **S2447**
23	SPIN1	Q9Y657	T31, **S124**, S241
24	KI20A	O95235	**S244**, S632, S677
25	AZI1	Q9UPN4	**S76**, **T93**, S202
26	EDC4	Q6P2E9	**S871**, T1075
27	CLAP1	Q7Z460	**S646**, S687, T730, S741
28	CK5P2	Q96SN8	**S547**, S1804
29	NUMA1	Q14980	T1879, S1883, S1887, S1901, **S1945**, S1969, S1991, T2007, S2026, S2047
30	ASPM	Q8IZT6	S149, S160, S170, **S270**, T503, T640, **S1103**, S3003, T3383, S3387, T3406, S3425
31	MACF1	Q9UPN3	S57, T362, S647, T671, S814, S1268, T1393, **S5009**, S5086, T5158, T5208, S5264, S5267, S5277, S5324, S5350, S5367, S5372, S5409, T5418, T5419
32	NIN	Q8N4C6	S109, T811, S812
33	CETN1	Q12798	S85, T102, S122, S170
34	1433G	P61981	S58, S59, S71, T145
35	HSP71	P08107	T425, T495, T502, S537
36	1433E	P62258	S59
37	2AAA	P30153	S146, T268, T568
38	CBX3	Q13185	**S93**
39	Q9BUB1	Q9BUB1	**S58**, **S99**
40	NUSAP	Q9BXS6	**S240**
41	TBB2C	P68371	**S322**, **S338**
42	DC1L2	O43237	**T441**
43	DC1L1	Q9Y6G9	**T213**, **T456**, **S487**
44	KNTC2	O14777	**S55**, **S62**
45	CND2	Q15003	**S70**
46	Q86T11	Q86T11	**S717**, **S752**
47	KIF23	Q02241	**S160**, **S911**, **S912**
48	MAP4	P27816-2	**T354**, **S863**
49	Q9BTE9	Q9BTE9	**T668**
50	MAP4	P27816	**S636**
51	SPAG5	Q96R06	**S362**
52	NUP98	P52948	**S618**
53	CASC5	Q8NG31	**S60**, **S765**
54	STK6	O14965	S155, T288
55	KAPCA	P17612	S339
56	CDC20	Q12834	S487, S492
57	CKAP5	Q14008	T1813, S1825, S1861, S1872, S1983, S1988
58	LATS1	O95835	S4, S37, S45, S58, S84, S142, S416, S629, S690
59	CTRO	O14578	S25, T119, S480, S496, S520, S582, S592, S652, S888, S942, T1018, T1174, S1568
60	TBB4	P04350	S176, T218
61	DCTN1	Q14203	S19, S351, T460, T481, T753, T950, S958, S974, T1044, T1152, S1153, T1210, T1249
62	CETN2	P41208	T102, S170
63	TP4A1	Q93096	S163
64	CBX1	P83916	S141, T169
65	DPOLB	P06746	S104
66	SEPT2	Q15019	S31, S234
67	WDR8	Q9P2S5	S10, T117
68	SEPT9	Q9UHD8	S324, T518
69	CDC23	Q9UJX2	S257
70	KIF22	Q14807	S244, T284, S389
71	KI18A	Q8NI77	S266
72	GCP3	Q96CW5	S92, S896, S904, S905
73	ANLN	Q9NQW6	T684, S753, S803, S937
74	CROCC	Q5TZA2	S676, S1069, S1084, T1109, S1187, S1204, S1218, S1569, S1575, S1591, S1619, S1624, S1784, T1881, S1900, S2004, S2005
75	CEP35	Q5VT06	S4, S17, T22, S67, S68, S76, T96, S232, S239, T255, S557, T577, S814, T831, T956, S974, S1143, T1307, T1658, T1680, S2161, S2737, S2744, S2752, T2766, T3100
76	DYH5	Q8TE73	T948, T974, T977, S996, S1013, S2942, S3335, T3474, T3504, T3769, T3791, S3824
77	SUMO1	P63165	S9
78	CDC2	P06493	T26
79	KC1A	P48729	T109, S237, S242
80	KC1D	P48730	S101, S229
81	TBG1	P23258	T288
82	TBB5	P07437	S48, S176, T218
83	NEK2	P51955	S29
84	FBX5	Q9UKT4	S94, S354, T395, S440
85	TCPA	P17987	T13
86	HS90B	P08238	S206
87	HS90A	P07900	S211
88	CDC27	P30260	T171
89	PP1G	P36873	S22
90	Q07161	Q07161	S336

Fig 2 | Ranking of the 90 substrate candidates for Aurora kinases. Known substrates of AurA or B are shown in purple. Experimentally tested candidates are shown in grey, with new confirmed substrates shown in green. Known direct or indirect AurA interactors are shown in orange and yellow, respectively. All accessible and conserved matches to the notP motif ([KR].[ST][^P]) are listed and known *in vivo* phosphorylation sites are marked in bold.

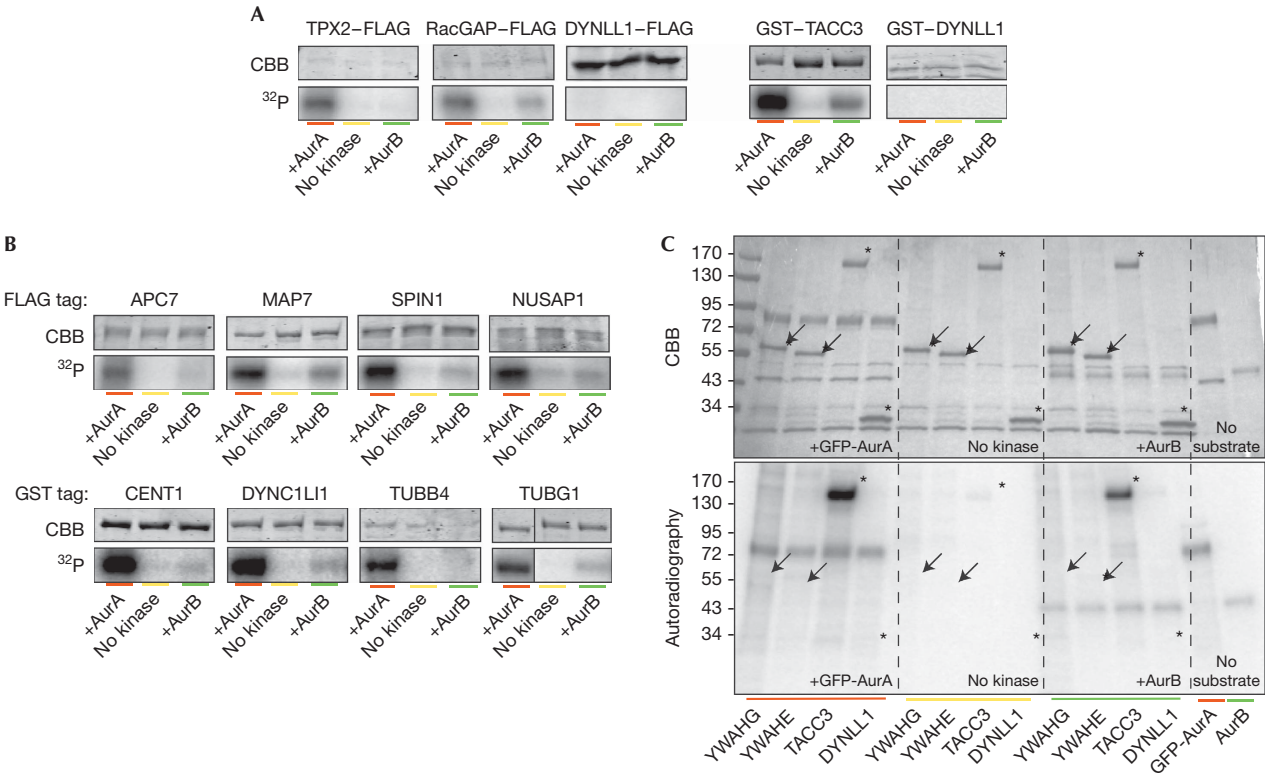

Fig 3 | Validation of candidate substrates by *in vitro* phosphorylation assays. (**A**) Specificity of the *in vitro* Aurora kinase assay tested on known AurA substrates and on a protein not containing the phosphorylation motif (DYNLL1, negative control). Substrates were expressed in human cells, and pulled down by their FLAG or GST tag. Precipitated proteins were mixed with AurA, AurB or only buffer in the presence of ^{32}P-ATP, then separated by SDS–PAGE and stained with Coomassie brilliant blue. Incorporated ^{32}P was visualized by autoradiography. The gel fragments shown belong to representative experiments repeated at least three times. Exposure time of the autoradiographs was adapted for each substrate to allow the visualization of the ^{32}P incorporation. (**B**) AurA phosphorylation assays were performed as in **A** on potential substrates from the candidate list. (**C**) Autoradiography (^{32}P) and Coomassie staining of an *in vitro* kinase assay gel, where YWHAG (14-3-3γ) and YWHAE (14-3-3ε) were tested in comparison with a positive (TACC3) and a negative control (DYNLL1). Arrows and asterisks, positions of candidate proteins. APC7, anaphase-promoting complex 7; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

Uncovering new functions for Aurora kinases

To explore the different roles of the putative Aurora substrates, we grouped them according to their cellular functions using the information in the UniprotKB database (Fig 5). This analysis showed that several of the new candidates clustered into groups, defining specific functions previously shown to be regulated by the Aurora kinases—for example, centrosome maturation and chromosome alignment. The clustering of potential substrates suggests that there might be a coordinated regulation of different proteins for any given function. Our analysis also indicates that the Aurora kinases might regulate yet more processes, such as sumoylation and protein degradation. Indeed, we found five different proteins of the APC-dependent degradation machinery in our candidate list, including APC7, which we have validated *in vitro*. Another interesting functional cluster contains proteins related to cilia, and a recent work has shown that AurA is essential for cilia reabsorption before the entry of quiescent cells into mitosis (Pugacheva *et al*, 2007). The putative phosphorylation of two cilia proteins—CROCC and DNAHC5—by AurA suggests a wider role for this kinase in the regulation of cilia function. The identification of new putative substrates for the Aurora kinases will enable not only the further investigation of some of their known functions, but also the examination of their potential regulatory role in other cellular processes.

CONCLUSIONS

We describe a new approach to identify candidate substrates for the Aurora kinases. Our *in vitro* results and the *in vivo* validation of NUSAP and CENP-E suggest that our method has predictive potential. As a consequence of our stringent filtering criteria—designed to maximize specificity—we might have excluded some Aurora substrates, the total number could therefore be more than 100. Although we have only applied our method to Aurora, it could be adapted to identify substrates of other kinases. To estimate its general applicability, we investigated how many of the 57 known mitotic kinases (Schmit & Ahmad, 2007) have enough phosphorylation data (at least ten different substrates) available from which to derive reliable phosphorylation patterns. We found

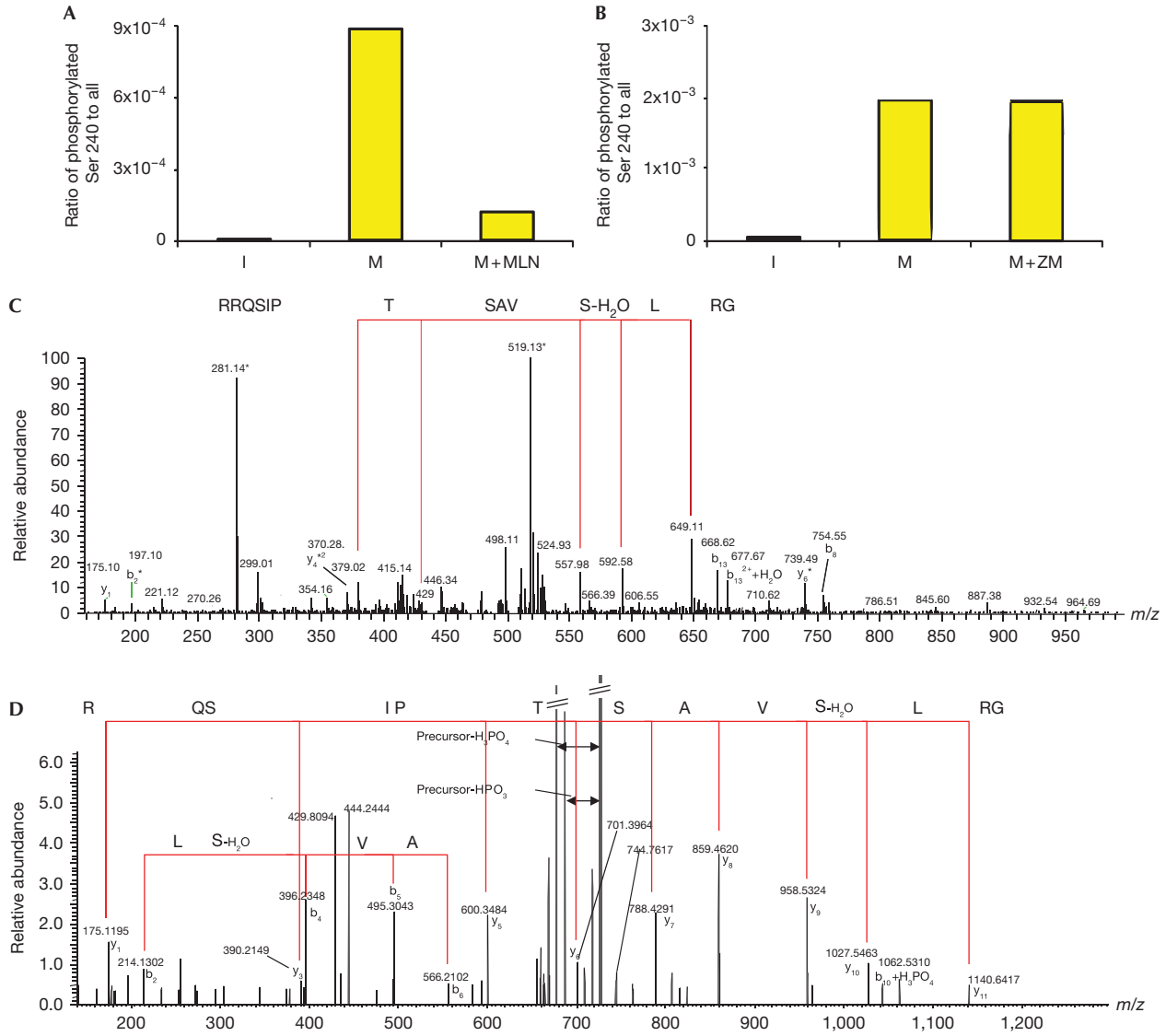

Fig 4 | NUSAP mitotic phosphorylation at Ser 240 correlates with Aurora A activity. Protein samples of FLAG–NUSAP immunoprecipitated from I, M and M + MLN or with M + ZM were analysed using LC-MS/MS, focusing on the predicted phosphorylated residue Ser 240. The histograms (**A**, **B**) show the calculated ratios based on peptides carrying the phosphorylated Ser 240 compared with all matched peptides containing this residue. (**C**, **D**) MS/MS spectra of two peptides matched the triply charged phosphorylated peptide GRLSphosVASTPISQRR and the doubly charged phosphorylated peptide GRLSphosVASTPISQR, respectively. In both spectra, y-ion series are marked with red lines indicating matched amino-acid sequences (**C**: doubly charged y-ion series and **D**: singly charged y-ion series) together with some b ions. The conversion of phosphorylated Ser 240 into dehydroalanine is marked in the spectra. I, interphase cells; LC-MS/MS, liquid chromatography-mass spectrometry; M, mitotic cells arrested with monastrol; M + MLN, mitotic cells co-treated with monastrol and MLN8237; M + ZM, monastrol and ZM447439.

that it would be possible to apply our strategy to 14 of them (25%). Considering all 113 human kinases implicated in mitosis—including the pleiotropic kinase families protein kinase A and mitogen-activated protein kinase—we can automatically use our strategy on 32 of them, increasing the potential applicability of the method to about 30%. Overall, we anticipate that the general approach presented here and the strategies to align functional motifs will help to decipher the many cellular functions that are regulated by kinases, including some that are yet to be discovered.

METHODS

All the methodological details referring to the biological data, computational analyses, protein expression and purification, *in vitro* kinase assays, immunoprecipitation of NUSAP from interphase and mitotic cells, and mass spectrometry analyses are provided in the figure legends and the supplementary information (online) accompanying the paper.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

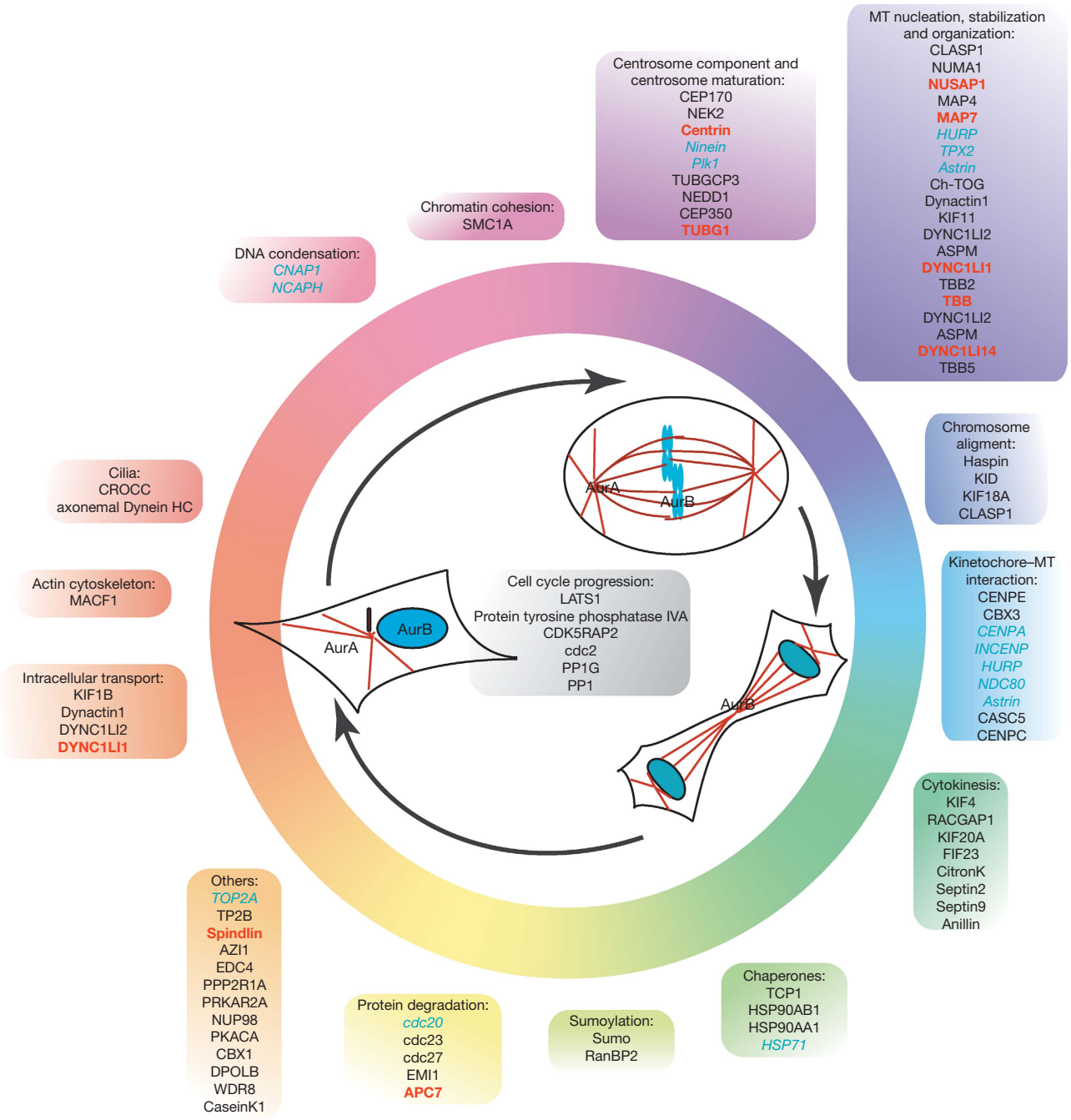

Fig 5 | Functional distribution of the predicted Aurora substrates. Known and candidate substrates classified according to their functions throughout the cell cycle. In the cell schemes, microtubules appear in red and DNA in blue. In the text boxes, blue, red and black indicate known, validated and proposed Aurora substrates, respectively.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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