Phosphorylation by Cyclin B–Cdk Underlies Release of Mitotic Exit Activator Cdc14 from the Nucleolus

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Budding yeast protein phosphatase Cdc14 is sequestered in the nucleolus in an inactive state during interphase by the anchor protein Net1. Upon entry into anaphase, the Cdc14 early anaphase release (FEAR) network initiates dispersal of active Cdc14 throughout the cell. We report that the FEAR network promotes phosphorylation of Net1 by cyclin-dependent kinase (Cdk) complexed with cyclin B1 or cyclin B2. These phosphorylations appear to be required for FEAR and sustain the proper timing of late mitotic events. Thus, a regulatory circuit exists to ensure that the arbiter of the mitotic state, Cdk, sets in motion events that culminate in exit from mitosis.

The nucleolus serves as a storage depot for regulatory factors including Mdm2, Pch2, Sir2, and Cdc14 (*1*). However, the mechanisms that underlie the release of proteins from nucleolar storage are unknown. In *Saccharomyces cerevisiae*, Cdc14 regulates anaphase and initiates a sequence of events culminating in mitotic cyclin-Cdk inactivation and exit from mitosis (*2*). Before anaphase, inactive Cdc14 is sequestered in the nucleolar RENT (regulator of nucleolar silencing and telophase) complex through its association with Net1 (*3*, *4*). At the metaphase-to-anaphase transition, anaphase-promoting complex (APC) promotes securin degradation, freeing active separase (Esp1) (*5*). Esp1 induces release of Cdc14 from Net1 by an unknown mechanism (*6*) that requires a group of proteins (Spo12, Slk19, and Cdc5) that together with Esp1 are referred to as the FEAR network (*7*). Cdc14 activity generated in this manner triggers multiple late M-phase events, including segregation of sister nucleoli, transfer of INCENP-Aurora B from kinetochores to the spindle midzone, coordination of the two segregation phases of meiosis, and timely progression to exit from mitosis (*7–10*). Subsequently, the mitotic exit network (MEN) sustains the release of active Cdc14 during late anaphase, thereby enabling exit from mitosis.

In contrast to other MEN mutants, Cdc14 disengages and remains free from Net1 at the *cdc14-1* block (*11*) and Net1 remains hyperphosphorylated in these cells (*3*). To evaluate whether this hyperphosphorylation might underlie cell cycle–regulated disruption of RENT, we purified Net1 from *cdc14-1* cells and mapped 19 sites of phosphorylation (table S1) (*12*).

To evaluate the importance of Net1 phosphorylation, we substituted for endogenous *NET1* a mutant allele (*net1-13m*) in which 13 phosphorylation sites within the N-terminal region were converted to alanine (table S1). We focused on N-terminal phosphorylation sites because deletion analysis revealed that amino acids 1 to 600 of Net1 were necessary and sufficient for proper nucleolar sequestration of Cdc14 in G_1 phase and its dispersal in anaphase (fig. S1). The mutant protein, Net1-13m, localized properly (*13*) and directed the nucleolar localization of Cdc14 (fig. S2A) but did not transiently release Cdc14 in early anaphase (Fig. 1A). To determine which mutations caused the FEAR-deficient phenotype of *net1-13m*, we constructed mutants lacking subsets of phosphorylation sites (fig. S2, A and B). Mutants lacking either three mapped Cdk consensus sites (*net1-3Cdk*) or all six Cdk sites within amino acids

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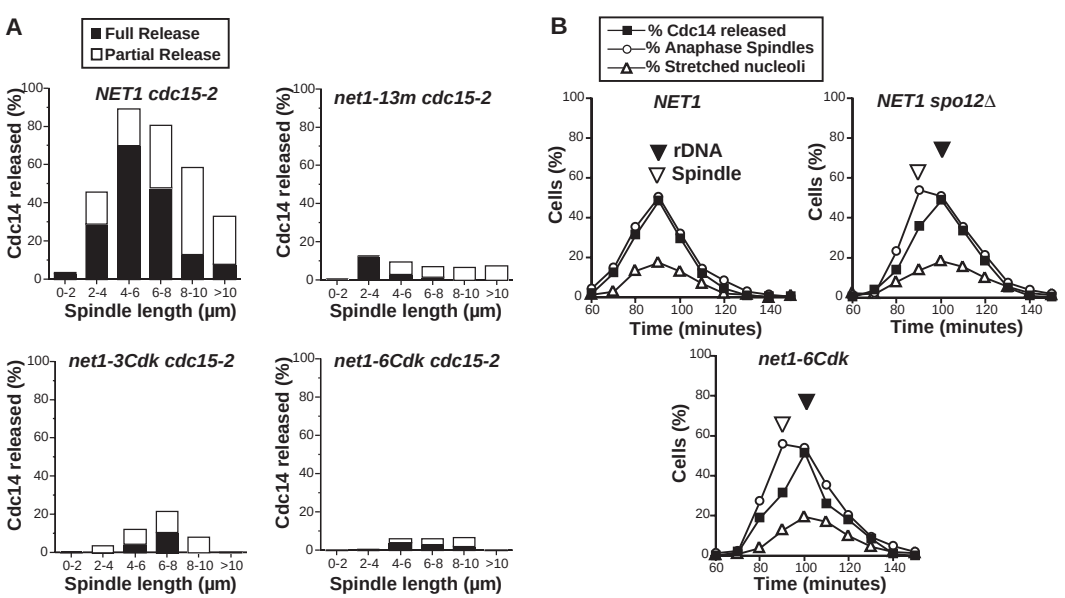

Fig. 1. Net1 phosphorylation site mutants are defective in release of Cdc14 from the nucleolus during early anaphase. (**A**) Quantitation of FEAR defect of Net1 phosphorylation site mutants. Synchronized *cdc15-2* cells carrying the indicated constructs were collected at 70 to 110 min after release from α factor and double-labeled with antibodies to Cdc14 and tubulin. Spindle length was measured and localization of Cdc14 was determined to be either nonnucleolar (full release) or partially nucleolar (partial release). More than 350 cells were counted for each panel. (**B**) Delayed Cdc14 release from the nucleolus and rDNA segregation in *net1-6Cdk* and *spo12*△ mutants. Synchronized cells collected up to 140 min after release from α factor were evaluated by immunofluorescence to monitor Cdc14 release from the nucleolus and segregation of the nucleolar antigen RPA190. Percentages of cells with stretched nucleoli were determined by counting cells in which the nucleolus had stretched between mother and daughter cells but had not segregated into two distinct masses and dividing by the total number of cells counted. Peak anaphase spindle and nucleolar segregation are indicated for each panel. More than 200 cells were counted for each time point.

1 to 341 (*net1-6Cdk*) almost completely recapitulated the phenotypes of *net1-13m* (Fig. 1A) (fig. S3A). These effects were specific, because another mutant (*net1-3Ax*) lacking three non-Cdk phosphorylation sites displayed only a minor reduction in FEAR (fig. S2C). Notably, most of the N-terminal Cdk sites are conserved among Net1 orthologs in other ascomycetes (fig. S4).

Although FEAR is typically assayed in the context of a MEN mutant such as *cdc15-2*, *spo12*△ single mutants exhibit a modest delay (~10 min) in release of Cdc14 from the nucleolus and exit from mitosis (*7*). Likewise, Cdc14 release from the nucleolus in *net1-6Cdk* was delayed by 10 min relative to elongation of anaphase spindles (Fig. 1B) (*14*). A third phenotype exhibited by *net1-3Cdk* and *net1-6Cdk* alleles is that they exacerbated the temperature-sensitive (*ts*) growth of MEN mutants *dbf2-2* and *cdc15-2*, respectively (fig. S3B). Taken together, these observations implicate *net1-3Cdk* and *net1-6Cdk* as FEAR mutants.

As a consequence of their delayed activation of Cdc14, FEAR network mutants exhibit delayed ribosomal DNA (rDNA) segregation and defects in coordination of meiosis I and II (*9*, *10*, *15*). We thus evaluated the ability of *net1-6Cdk* mutants to segregate rDNA and form meiotic spores. Mutant *spo12*△ and *net1-6Cdk* cells exhibited a 10-min delay in rDNA segregation during mitosis (Fig. 1B). Also, whereas >90% of nitrogen-starved wild-type diploid cells produced tri- or tetranucleate asci, homozygous *net1-6Cdk* diploids produced 43% binucleate and 57% tri- or tetranucleate asci, most of which were trinucleate (*13*). This meiotic defect closely resembles that of *slk19*△ mutants (*10*).

Recombinant Clb2-Cdk, but not an equivalent amount of Clb5-Cdk activity, incorporated radiolabel from [γ-^{32}P]adenosine triphosphate (ATP) into Net1 (Fig. 2A). To study phosphorylation of Net1 by Cdk in greater detail, we prepared antibodies reactive to the phosphorylation sites mutated in *net1-3Cdk*. All three antibodies (to PP-A, PP-B, and PP-C) reacted with Net1 isolated from arrested *cdc14-1* cells but not with either Net1-3Cdk (fig. S5A) or wild-type Net1 from α factor–arrested cells (Fig. 2C, lane 1). Moreover, treatment with Clb2-Cdk rendered Net1 reactive toward antibodies to PP-B (Fig. 2B) and PP-C (fig. S5B). In most subsequent experiments, we used antibody to PP-B (which detects phosphorylated Thr^{212}) because it yielded a better signal in Western blot analyses.

If Clb2-Cdk (and the closely related Clb1-Cdk) is indeed a physiological Net1 kinase, Net1 phosphorylation should be diminished or delayed in *clb2*△ or *clb1*△ *clb2*△ mutants. In *clb2*△ cells released from a G_1 cell cycle block, appearance of the PP-B epitope and Cdc14 release were delayed by ~30 min (Fig. 2C) (fig. S5E). Moreover, a synchronized culture of *clb1*△ *clb2*△ *GAL1p-CLB2* cells depleted of the majority of Clb2 arrested cell cycle progression in late anaphase or telophase (fig. S5F) without detectable phosphorylation of Net1 on Thr^{212} (Fig. 2D) or release of Cdc14 from the nucleolus (Fig. 2E) (figs. S5F and S6A). Phosphorylation of Net1 on Thr^{212} was also eliminated upon chemical inhibition of Cdc28-as1 kinase activity (*16*, *17*) in cells released from a metaphase block (Fig. 2F). Finally, *clb2*△ mimicked FEAR network mutants; *cdc15-2 clb2*△ cells failed to exhibit FEAR (Fig. 2E) (fig. S6A), *clb2*△ enhanced the *ts* phenotype of *cdc15-2* (fig. S6B) and *dbf2-2* (*13*) mutants, and *clb2*△ was lethal in combination with *cdc14-1*, *cdc5-1*, or *msd2-1* (*13*, *18*). The synthetic phenotype of *cdc15-2 clb2*△ appeared to arise from an exacerbation of the MEN defect because the terminal phenotype remained a late-anaphase arrest (fig. S6A) (*13*), even though the double mutant cells exhibited a 30-min delay at metaphase, as expected for a *clb2*△ mutant (fig. S6C).

To determine whether regulation of Clb2-Cdk might normally govern the timing of FEAR, we tested whether Clb2 overproduction was sufficient to activate ectopic release of Cdc14 from the nucleolus. Whereas endogenous Clb2 did not sustain Thr^{212} phosphorylation in metaphase-arrested cells (Figs. 2F and 3A), overexpression of a stabilized form of Clb2 (CLB2C$_2$DK$_{100}$) (*19*) promoted Net1 phosphorylation and dispersal of Cdc14 in metaphase (Fig. 3, A and B) but not G_1 phase cells (*13*). Thus, phosphorylation of Net1 by Clb2-Cdk in vivo may require other factors that are cell cycle–regulated. The ability of overexpressed Clb2 to induce premature release of Cdc14 from the nucleolus in metaphase prompted us to test whether Clb2-Cdk can disassemble RENT in vitro. Phosphorylation of bacterially expressed Net1 (amino acids 1 to 600) with recombinant Clb2-Cdk blocked its binding to Cdc14 (Fig. 3C). In addition, Net1-Cdc14 complexes immunoprecipitated from yeast cells were dis-

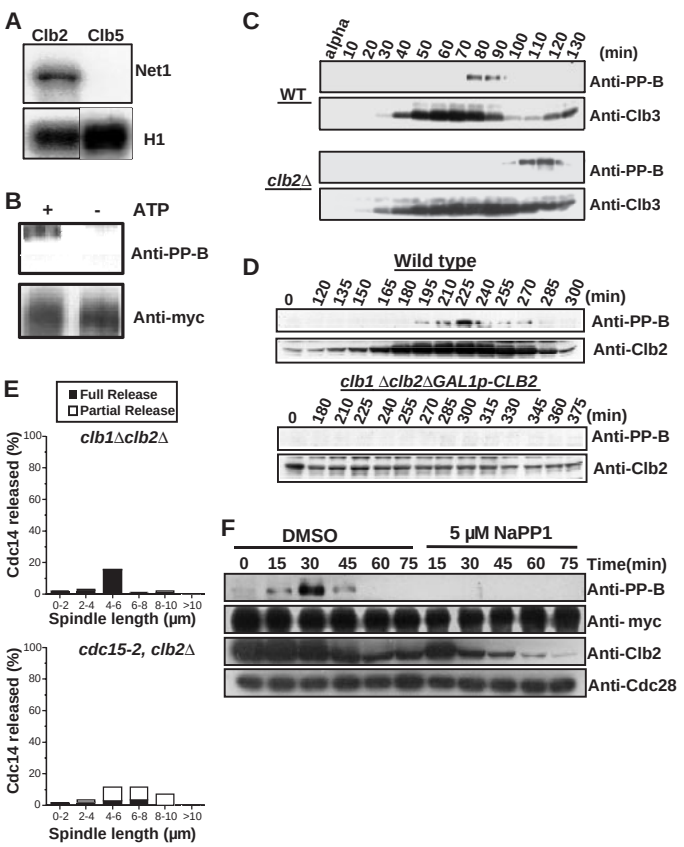

Fig. 2. Clb2-Cdk is necessary and sufficient for phosphorylation of Net1 on Cdk sites. (**A**) Net1 is a substrate for Clb2-Cdk. Net1 or histone H1 incubated with [γ-^{32}P]ATP plus Clb2-Cdk or Clb5-Cdk were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) and subjected to autoradiography. (**B**) Clb2-Cdk phosphorylates Net1 on physiological sites. Net1-myc9 immunoprecipitated from α factor–arrested cells was treated with Clb2-Cdk with (+) or without (–) ATP and immunoblotted with antibodies to PP-B or myc. (**C**) Phosphorylation of Net1 on Cdk sites delayed in *clb2*△. Cultures were synchronized in α factor and released before sampling with the indicated antibodies. Clb3 is a marker for cell cycle progression. Loading controls and Clb2 amounts are shown in fig. S5C. (**D**) Phosphorylation of Net1 on Cdk sites abolished in *clb1*△ *clb2*△ cells. Cells grown in galactose medium were synchronized by elutriation (*21*, *22*) and inoculated into glucose medium to extinguish Clb2 expression before sampling. Loading controls are shown in fig. S5D. (**E**) FEAR defect of *clb1*△ *clb2*△ and *cdc15-2 clb2*△ cells. Cells synchronized as in (C) (*cdc15-2 clb2*△) or (D) (*clb1*△ *clb2*△ *GAL1p-CLB2*) were evaluated for FEAR as described in Fig. 1A. (**F**) *NET1-myc9 cdc28-as1* cells were released from a metaphase block in the presence of 5 μM NaPP1 (specific inhibitor of *cdc28-as1*) or dimethyl sulfoxide (DMSO; vehicle) before sampling with the indicated antibodies.

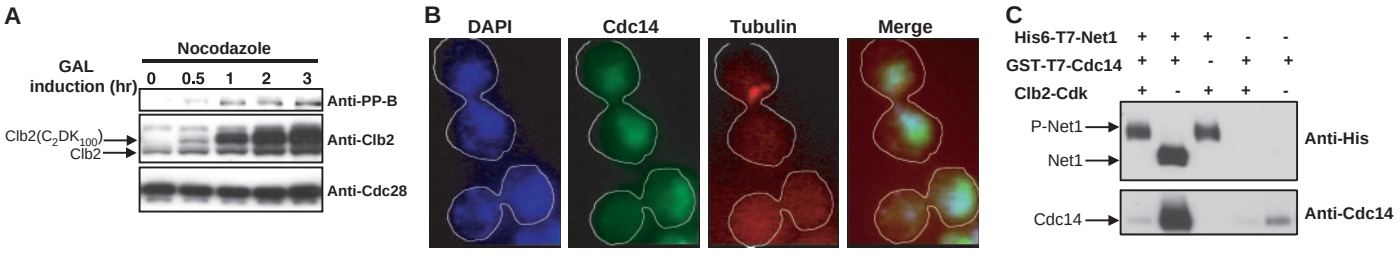

Fig. 3. Clb2-Cdk disrupts sequestration of Cdc14 by Net1. (**A** and **B**) Overexpression of stabilized Clb2 (Clb2-C_2DK_{100}) from the *GAL1* promoter promotes Net1 phosphorylation on Thr^{212} (A) and dispersal of Cdc14 from the nucleolus (B; magnification, 100×). Net1 phosphorylation and Cdc14 localization were evaluated as described (Fig. 2C) (fig. S3A). In (B), the outlines of cells are drawn; ~40% of cells released Cdc14. (**C**) Phosphorylation of Net1 (amino acids 1 to 601) by Clb2-Cdk disrupts Cdc14 binding in vitro. Immobilized His6-T7-Net1 was treated (+) or not (–) with Clb2-Cdk before incubation with GST-T7-Cdc14. Bead-bound proteins were fractionated by SDS-PAGE and blotted with the indicated antibodies. (**D**) (Net1-6Cdk)–Cdc14 complex is refractory to disassembly by Clb2-Cdk in vitro. RENT complexes retrieved from the indicated genotypes by immunoprecipitation were treated with Clb2-Cdk protein kinase (*23*). Bead and supernatant fractions were resolved by SDS-PAGE and immunoblotted with antibodies to Cdc14.

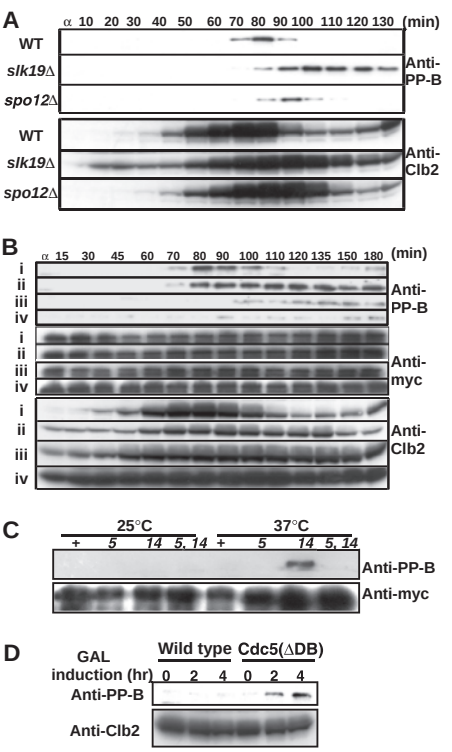

Fig. 4. Net1 phosphorylation on Thr^{212} is modulated by the FEAR network. (**A**) Thr^{212} phosphorylation is delayed in *slk19*△ and *spo12*△ mutants. Extracts of cells released from α factor block and sampled at the indicated time points were fractionated by SDS-PAGE and immunoblotted with the indicated antibodies. Loading controls are shown in fig. S7A. (**B**) Thr^{212} phosphorylation is nearly eliminated upon simultaneous inactivation of the FEAR and mitotic exit networks. Same as (A), except that the strains analyzed were (i) wild type, (ii) *cdc15-2*, (iii) *cdc15-2 spo12*△, and (iv) *cdc15-2 slk19*△. Antibody to myc was used to monitor Net1-myc9. Loading controls are shown in fig. S7B. (**C**) Net1 phosphorylation on Thr^{212} is dependent on Cdc5. Same as (A), except that asynchronous cultures were sampled 3 hours after shift to 37°C. Lanes: +, wild type; 5, *cdc5-1*; 14, *cdc14-1*; 5, 14, *cdc5-1 cdc14-1*. Loading controls and Clb2 amounts are shown in fig. S7D. (**D**) Overexpressed Cdc5 promotes Net1 phosphorylation on Thr^{212} in metaphase-arrested cells. Wild-type and *GAL1p-CDC5(△DB)* cells were arrested in metaphase, induced with galactose for 0 to 4 hours, and evaluated as described in (A). Loading controls and Cdc5△DB induction levels are shown in fig. S7E.

sociated when incubated with Clb2-Cdk, whereas (Net1-6Cdk)–Cdc14 complexes were not (Fig. 3D).

Together, these results provide strong evidence that phosphorylation of Net1 on a set of N-terminal Cdk sites by Clb1-Cdk or Clb2-Cdk is a key event that underlies FEAR. We were therefore surprised that Clb2 accumulation preceded the PP-B epitope by at least 10 to 20 min in synchronized cells (Fig. 2, C and E) (fig. S5C). Because Clb2-associated protein kinase activity mirrors Clb2 antigen (*7*), phosphorylation of Net1 by Clb2-Cdk appears to be regulated. We hypothesized that the FEAR network promotes Net1 phosphorylation by Clb2-Cdk in early anaphase. To test this hypothesis, we monitored the PP-B epitope in synchronized wild-type, *slk19*△, and *spo12*△ strains. The timing of Net1 phosphorylation was delayed in both mutants by 10 to 20 min (Fig. 4A), which correlated with the delay in Cdc14 release and rDNA segregation seen in *spo12*△ (Fig. 1B). These data suggest that the FEAR network enables phosphorylation of Net1 by Clb1-Cdk or Clb2-Cdk in early anaphase, but that these phosphorylations (like other events triggered by the FEAR network) can be mobilized independently in late anaphase by the MEN. Consistent with this idea, phosphorylation of both Net1 on Thr^{212} (Fig. 4B) and FEAR (fig. S7C) were nearly eliminated in *cdc15-2 spo12*△ and *cdc15-2 slk19*△ double mutant cells.

Protein kinase Cdc5 (polo) influences the phosphorylation state of Net1 and promotes release of Cdc14 from the nucleolus, primarily by an indirect mechanism (*20*). Cdc5, like Spo12 and Slk19, appears to act upstream of Clb-Cdk phosphorylation of Net1, because PP-B epitope formation was absent in *cdc14-1 cdc5-1* cells (Fig. 4C) and overexpression of stabilized (△DB) Cdc5 promoted phosphorylation of Thr^{212} in nocodazole-arrested cells (Fig. 4D).

Cdc14 released through the action of the FEAR network plays a key role in organizing multiple events during early anaphase (*7*–*10*). Our results show that the FEAR network acts in part by stimulating phosphorylation of Net1 by Clb-Cdk, which directly weakens the interaction of Cdc14 with Net1, allowing for Cdc14 activation and mitotic exit.

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Supporting Online Material
www.sciencemag.org/cgi/content/full/305/5683/516/DC1
Materials and Methods
Figs. S1 to S7
Tables S1 and S2
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Functional Adaptation of BabA, the *H. pylori* ABO Blood Group Antigen Binding Adhesin

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Adherence by *Helicobacter pylori* increases the risk of gastric disease. Here, we report that more than 95% of strains that bind fucosylated blood group antigen bind A, B, and O antigens (generalists), whereas 60% of adherent South American Amerindian strains bind blood group O antigens best (specialists). This specialization coincides with the unique predominance of blood group O in these Amerindians. Strains differed about 1500-fold in binding affinities, and diversifying selection was evident in *babA* sequences. We propose that cycles of selection for increased and decreased bacterial adherence contribute to *babA* diversity and that these cycles have led to gradual replacement of generalist binding by specialist binding in blood group O–dominant human populations.

The gastric pathogen *Helicobacter pylori* causes chronic inflammation, which may progress to peptic ulceration and gastric cancer (*1*). *H. pylori* expresses adhesins that confer intimate adherence to the gastric epithelium, where the bacteria can gain easy access to nutrients from host tissues. Both fucosylated glycoproteins and sialylated glycolipids have been shown to be binding sites for *H. pylori* in the gastric epithelium (*2*, *3*). The South American *H. pylori* strain P466 has been shown to bind the fucosylated H1 and Lewis b antigens (blood group O phenotype) but not the A–Lewis b antigen (blood group A phenotype) (*2*). These fucosylated blood group antigens are highly expressed in gastrointestinal epithelium (*4*). The H antigen is the carbohydrate structure that defines blood group O phenotype in the ABO blood group system. The Lewis b antigen (Leb), which is difucosylated, is formed by addition of a branched fucose (Fuc) residue to H1. The antigens that define blood group A and B phenotypes and corresponding antigens in the Lewis blood group system are formed by terminal N-acetylgalactosamine (GalNAc) or galactose (Gal) substitutions of H1 and Leb [A and A–Lewis b (ALeb) and B–Lewis b (BLeb) antigens, respectively] (Fig. 1A, fig. S1, and table S1). Epidemiologically, individuals of blood group O phenotype are particularly prone to peptic ulcer disease (*5*). Binding of *H. pylori* to H1 and Leb is mediated by the blood group antigen–binding adhesin BabA, an outer membrane protein (*6*) expressed by most disease-causing *H. pylori* strains (*7*).

The present study began with an observation that binding of *H. pylori* strain CCUG17875 (17875) to the gastric mucosa could be blocked by pretreatment with soluble ALeb in addition to Leb. However, adherence of strain P466 could not be blocked by pretreatment with ALeb even though it was inhibited by Leb (Fig. 1B and fig. S2). The binding specificities of several *H. pylori* strains were then studied with the use of soluble radiolabeled glycoconjugates (*8*). The blood group antigens used were all natural oligosaccharides, except for BLeb, which was synthesized chemically. Strains 17875 and J99 bound A, ALeb, BLeb, H1, and Leb, although subsequent tests indicated they did not bind the related Forssman antigen (with GalNAcα1.3 but not Fucα1.2) (*9*). By contrast, P466 only bound H1 and Leb (Fig. 1C and table S2). Similarly, with glycosphingolipids (GSLs) (*8*), strains 17875 and J99 each bound the difucosylated Leb, ALeb, and BLeb GSLs, whereas strain P466 only bound Leb GSL. Binding to monofucosylated GSLs was very weak or was observed only inconsistently. None of the strains bound to fucosylated antigens in the closely related type-2 core chain (fig. S3 and table S3). Consequently, we consider P466 to be a specialist strain of *H. pylori* and the other two to be generalists, able to tolerate bulky Gal and GalNAc end groups.

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