Supplemental Figure 1. Summary of Mass Spec results for CENP-L and CENP-N pulldowns and reference images for cell cycle analysis of CCAN protein localization.

A. Representative images of CENP-N throughout the cell cycle as quantified with the cell line expressing GFP tagged-CENP-N at the endogenous locus. Shown is DNA (Hoechst) and GFP at each cell cycle stage. GFP images are scaled relative to S phase signal. Representative images of each cell cycle stage marker are also shown: Microtubules (G1/prophase/mitosis); PCNA (S-phase-specifically cells with the mid-S phase PCNA stain (Schonenberger et al., 2015), Cyclin B (G2-).

B-C. Summary of proteins identified by mass spectrometry after each indicated immunoprecipitation. The proteins listed are all other inner kinetochore components with the corresponding peptide count, #PSMs, and % Sequence coverage. The bait for each IP is highlighted in red. In CENP-N chart values for both S phase enrichment and Mitosis enrichment are shown. PSM=peptide spectrum match

D. Table representing the non-CDK sites identified by our mass spec analysis and previously reported in the literature.

Supplemental Figure 2. CDK consensus sites in CENP-L and CENP-N are evolutionarily conserved.

A. Sequence alignments for the SP/TP sites found throughout the CENP-N sequence. SP/TP sites are highlighted in red. Sequence alignments generated with UniProt alignment.

B. Sequence alignments for the SP/TP sites found throughout the CENP-L sequence. SP/TP sites are highlighted in red. Sequence alignments generated with UniProt alignment.

Supplemental Figure 3. Characterization of CENP-N and CENP-L inducible knock-out cell lines

A. Representative images of GFP-CENP-N^{CDK-5A} localization in the CENP-N inducible knock out (iKO)cell lines. Top panel shows localization in the absence of Cas9 expression. The bottom panel shows localization following 5-day induction of Cas9 expression and therefore depletion of endogenous protein. Scale bar 5μm.

B. Fluorescence intensity quantification of CENP-C, CENP-K, and CENP-T at the kinetochore following knockdown of endogenous protein in the CENP-N replacement cell lines. The mean fluorescence intensity measured in each cell line are graphed as a ratio of the average fluorescence intensity measured in the wild type CENP-N replacement. For each condition, fluorescence intensity was measured from at least 40 kinetochores in 5 different cells.

C. Fluorescence intensity quantification of CENP-C, CENP-K, and CENP-T at the kinetochore following knockdown of endogenous protein in the CENP-L replacement cell lines. The mean fluorescence intensity measured in each cell line are graphed as a ratio of the average fluorescence intensity measured in the wild type CENP-L replacement. For each condition, fluorescence intensity was measured from at least 40 kinetochores in 5 different cells.
Supplemental Figure 4. Assessment of the recruitment of other CCAN proteins to the ectopically targeted CENP-L and CENP-N

A. Representative immunofluorescence images of cells with ectopically targeted CENP-N, using antibodies against other CENP proteins to detect their recruitment to the lacO array. Images are deconvolved and max projected. Scale bar is 5 μm.

B. Representative immunofluorescence images of cells with ectopically targeted CENP-N, using antibodies against other CENP proteins to detect their recruitment to the lacO array. Images are deconvolved and max projected. Scale bar is 5 μm.

Supplemental Figure 5. Localization of N-terminal fragments of CENP-N and mutants.

A. Representative images of the localization behavior of transiently expressed CENP-N\textsuperscript{1-212} fragments and mutants in HeLa cells. Constructs were transfected 48 hours before cells were fixed. Images were deconvolved and max projected. Scale bar is 5 μm.

B. Representative images of CENP-N truncations and mutants in interphase and mitosis demonstrating their similar localization behaviors. GFP-tagged constructs are transiently expressed. Images are deconvolved and max projected. Scale bar is 5 μm.

Supplemental Figure 6. Transfection of CENP-N phosphomutants in cell lines stably expressing CENP-L or CENP-L-CDK-5A.

A. Quantification of fluorescence intensities for recruitment of TdTomato-tagged CENP-L or CENP-L\textsuperscript{CDK-7\textdagger} to the LacI-CENP-N/LacI-CENP-N\textsuperscript{CDK-5\textdagger} focus. Each dot represents the ratio between TdTomato:GFP fluorescence at a single foci. Data combined from 2 individual experiments. Error bars represent standard deviation. Unpaired two-tailed t test was performed. P values from left to right: ****=<0.0001, *=0.0316, **** =0.0001, ns=0.3306. N for each experiment from left to right: N=10, N=9, N=10, N=10, N=8, N=10.

B. Quantification of fluorescence intensities for recruitment of TdTomato-tagged CENP-L or CENP-L\textsuperscript{CDK-7\textdagger} to the LacI-CENP-N/LacI-CENP-N\textsuperscript{CDK-5\textdagger} foci. Each dot represents the ratio between TdTomato:GFP fluorescence at a single foci. Data combined from 3 individual experiments. Error bars represent standard deviation. Unpaired two-tailed t test was performed. P values from left to right: ****=<0.0001, ns=0.6362. N values from left to right: N=18, N=18, N=17, N=17.

C. Representative immunofluorescence images of cells in each condition. Cell lines are stably expressing mScarlett-CENP-L or mScarlett-CENP-L\textsuperscript{CDK-7\textdagger}, both cell lines were transiently transfected with plasmids expressing either GFP-CENP-N or GFP-CENP-N\textsuperscript{CDK-5\textdagger}. Cells were transfected 48 hours post dox induction of Cas9. Cells were fixed and processed for immunofluorescence 48 hours post transfection. Scale bar is 5 μm.

D. Representative immunofluorescence images of cells in each condition. Cell lines are stably expressing GFP-CENP-N or GFP-CENP-N\textsuperscript{CDK-5\textdagger}, both were transiently transfected with plasmids expressing TdTomato-CENP-L\textsuperscript{NtermCDK-4\textdagger} or TdTomato-CENP-L\textsuperscript{CtermCDK-7\textdagger}. Cells were transfected 48 hours post dox induction of Cas9. Cells were fixed and processed for immunofluorescence 48 hours post transfection. Images are deconvolved and max projected. Scale bar is 5 μm.

E. Representative immunofluorescence images of cells in each condition. Cell lines are stably expressing mScarlett-CENP-L or mScarlett-CENP-L\textsuperscript{CDK-7\textdagger}, both were transiently
transfected with plasmids expressing GFP-CENP-N\textsuperscript{NtermCDK-2A} or GFP-CENP-N\textsuperscript{CtermCDK-3A}. Cells were transfected 48 hours post dox induction of Cas9. Cells were then fixed and processed for immunofluorescence 48 hours post transfection. Images are deconvolved and max projected. Scale bar is 5 \( \mu \text{m} \).
Supplemental Figure 1

A

DNA

CENP-GFP

Cell Cycle Marker

Microtubules
PCNA
Cyclin B

G1
S phase
G2
Prophase
Metaphase

B

Anti-GFP-CENPN IP
Mitosis v. S phase

| Protein | # Peptides S phase/Mitosis | # PSM S phase/Mitosis | % Sequence Coverage S phase/Mitosis |
|---------|----------------------------|-----------------------|------------------------------------|
| CENP-N  | 27/29                      | 196/445               | 64/82%                             |
| CENP-L  | 4/21                       | 16/157                | 13/66%                             |
| CENP-C  | -4/9                       | -/9                   | -/7%                               |
| CENP-H  | 1/16                       | 5/97                  | 12/65%                             |
| CENP-I  | 1/17                       | 2/68                  | 2/30%                              |
| CENP-K  | 1/13                       | 1/54                  | 7/58%                              |
| CENP-M  | -7/5                       | -/28                  | -/35%                              |
| CENP-T  | 3/14                       | 5/34                  | 11/40%                             |
| CENP-W  | 1/2                        | 1/2                   | 10/30%                             |
| CENP-S  | 2/3                        | 2/15                  | 10/20%                             |
| CENP-X  | 1/4                        | 2/16                  | 9/64%                              |
| CENP-O  | 1/2                        | 3/4                   | 8/9%                               |
| CENP-P  | 1/5                        | 3/16                  | 3/22%                              |
| CENP-Q  | 1/7                        | 1/15                  | 5/33%                              |
| CENP-U  | 1/6                        | 3/23                  | 2/15%                              |
| CENP-R  | -7/3                       | -/16                  | -/16%                              |
| CENP-F  | -/2                        | -/2                   | -/1%                               |

C

Anti-GFP-CENPL IP
(Mitotically Enriched)

| Protein | # Peptides | # PSM | % Sequence Coverage |
|---------|------------|-------|---------------------|
| CENP-N  | 23         | 141   | 68%                 |
| CENP-L  | 17         | 152   | 54%                 |
| CENP-C  | 22         | 51    | 36%                 |
| CENP-H  | 16         | 52    | 64%                 |
| CENP-I  | 18         | 60    | 31%                 |
| CENP-K  | 10         | 31    | 57%                 |
| CENP-M  | 10         | 49    | 85%                 |
| CENP-T  | 13         | 36    | 41%                 |
| CENP-W  | 3          | 13    | 39%                 |
| CENP-S  | 5          | 15    | 28%                 |
| CENP-X  | 6          | 21    | 64%                 |
| CENP-O  | 5          | 10    | 20%                 |
| CENP-P  | 6          | 15    | 24%                 |
| CENP-Q  | 9          | 15    | 41%                 |
| CENP-U  | 5          | 18    | 15%                 |
| CENP-R  | 4          | 8     | 23%                 |
| CENP-F  | 2          | 3     | 1%                  |

D

| Protein | Residue | Sequence | # PSM | Xcorr | Source               |
|---------|---------|----------|-------|-------|----------------------|
| CENP-N  | S226    | SLGLDINMDSR | 7     | 3.71 | Mitotic-IP/MS (This Study) |
|         |         | RSLGLDINMDSR |   | 3.26 | Sharma, K., et. al. 2014 |
|         |         | QTFETHNSTTPLQERSLGLDINMDSRHIHENIVEKERVQR |   |       | Kettenbach, A., et. al. 2011 |
| CENP-N  | S235    | TPLGERSLGLDINMDRSRIHENIVEKERVQR |   |       |                      |
| CENP-N  | S277    | KSLGNSILAEERPLRC | 2.73 |       |                      |
| CENP-N  | S282    | SLGNSILAEERPL | 3     | 3.36 |                      |
|         |         | SLGNSILAER | 2     | 2.38 |                      |
|         |         | KSLGNSILAEERPLRC |   | 3.73 |                      |
|         |         | AQYKLETKFSGLGNSILAEERPLRCLIKF |   |       |                      |
Supplemental Figure 2

**A Sequence alignment CENP-N**

**HUMAN:**
```
| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 |
|----|----|----|----|----|----|----|----|----|----|----|
| A  | B  | C  | D  | E  | F  | G  | H  | I  | J  | K  |
```

**MOUSE:**
```
| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 |
|----|----|----|----|----|----|----|----|----|----|----|
| A  | B  | C  | D  | E  | F  | G  | H  | I  | J  | K  |
```

**BOVINE:**
```
| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 |
|----|----|----|----|----|----|----|----|----|----|----|
| A  | B  | C  | D  | E  | F  | G  | H  | I  | J  | K  |
```

**XENOPUS:**
```
| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 |
|----|----|----|----|----|----|----|----|----|----|----|
| A  | B  | C  | D  | E  | F  | G  | H  | I  | J  | K  |
```

**CHICKEN:**
```
| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 |
|----|----|----|----|----|----|----|----|----|----|----|
| A  | B  | C  | D  | E  | F  | G  | H  | I  | J  | K  |
```

**Sequence alignment CENP-L**

**HUMAN:**
```
| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 |
|----|----|----|----|----|----|----|----|----|----|----|
| A  | B  | C  | D  | E  | F  | G  | H  | I  | J  | K  |
```

**MOUSE:**
```
| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 |
|----|----|----|----|----|----|----|----|----|----|----|
| A  | B  | C  | D  | E  | F  | G  | H  | I  | J  | K  |
```

**BOVINE:**
```
| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 |
|----|----|----|----|----|----|----|----|----|----|----|
| A  | B  | C  | D  | E  | F  | G  | H  | I  | J  | K  |
```

**XENOPUS:**
```
| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 |
|----|----|----|----|----|----|----|----|----|----|----|
| A  | B  | C  | D  | E  | F  | G  | H  | I  | J  | K  |
```

**CHICKEN:**
```
| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 |
|----|----|----|----|----|----|----|----|----|----|----|
| A  | B  | C  | D  | E  | F  | G  | H  | I  | J  | K  |
```

Supplemental Figure 3

A. GFP-CENPN-CDK5A in CENPN iKO

B. CENP-C Levels in CENP-N Replacement Cell Lines

C. CENP-C Levels in CENP-L Replacement Cell Lines
Supplemental Figure 6

A

Quantification of TdTom-CENPL\textsuperscript{CDK-5D} and to LacI-CENPN\textsuperscript{CDK-7A}

B

Quantification of TdTom-CENPL and CENPL\textsuperscript{CDK-7A} to LacI-CENPN and CENPN\textsuperscript{CDK-5A}

C

DNA  GFP  RFP

D

DNA  GFP  RFP

E

DNA  GFP  RFP
