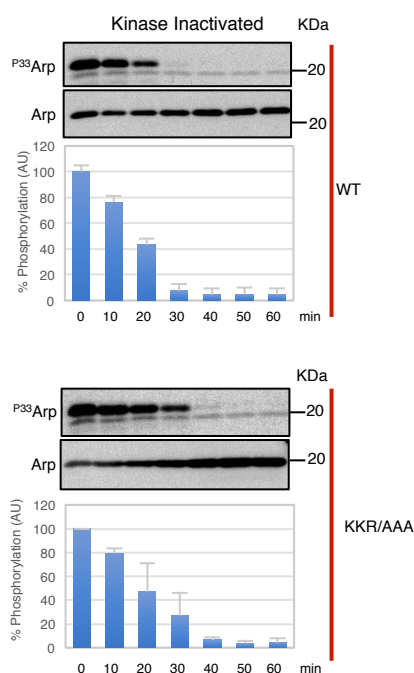
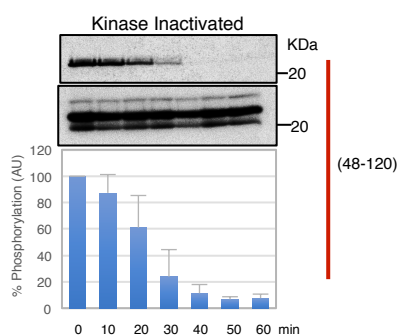


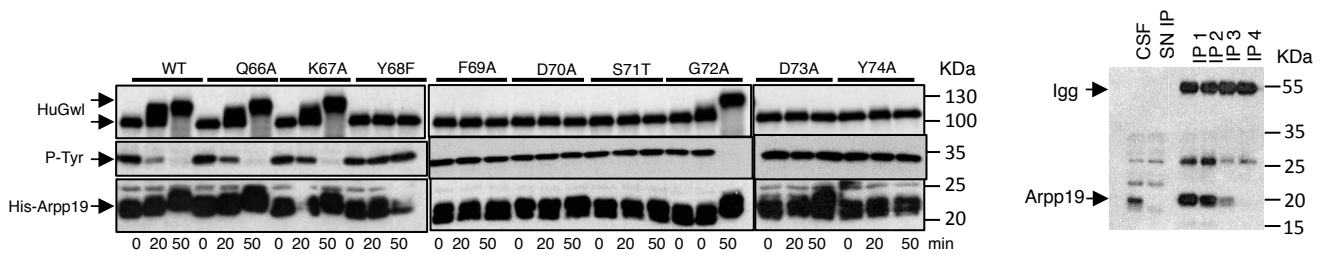
**A****B**

**Supplementary Figure 1.** Basic residues flanking the DSG motif positively modulate S71 dephosphorylation.

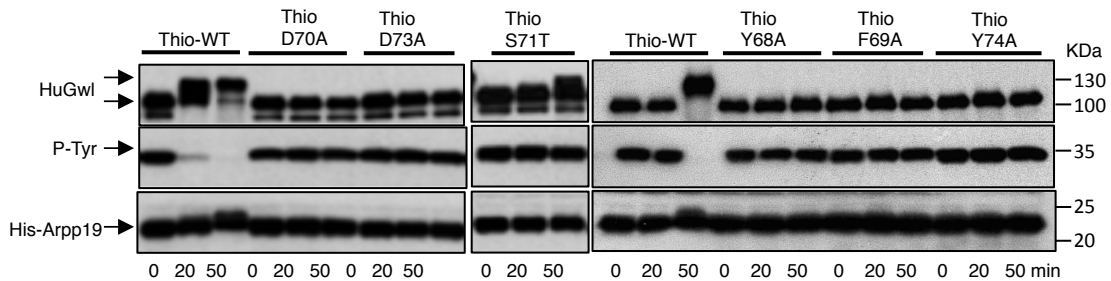
**(A)** Dephosphorylation of the wildtype and the KKR/AAA mutant form of Arpp19 was performed three times in kinase-inactivated extracts as indicated in material and methods at different kinetics between 0 and 60 minutes. Levels of Arpp19 and S71 phosphorylation were analysed by western blot and autoradiography and quantified by ImageJ. The mean percentage of the  $^{33}\text{P}$ -Arpp19/ Arpp19 ratio remaining at each time-point respect to time 0 min was calculated and represented as bar graphs +/- SD; n=3 biological independent samples.

**(B)** As for (A) except that the (48-120) mutant form of Arpp19 was used. The mean percentage of the  $^{33}\text{P}$ -Arpp19/ Arpp19 ratio remaining at each time-point respect to time 0 min was calculated and represented as bar graphs +/- SD; n=3 biological independent samples.

**A**



**B**

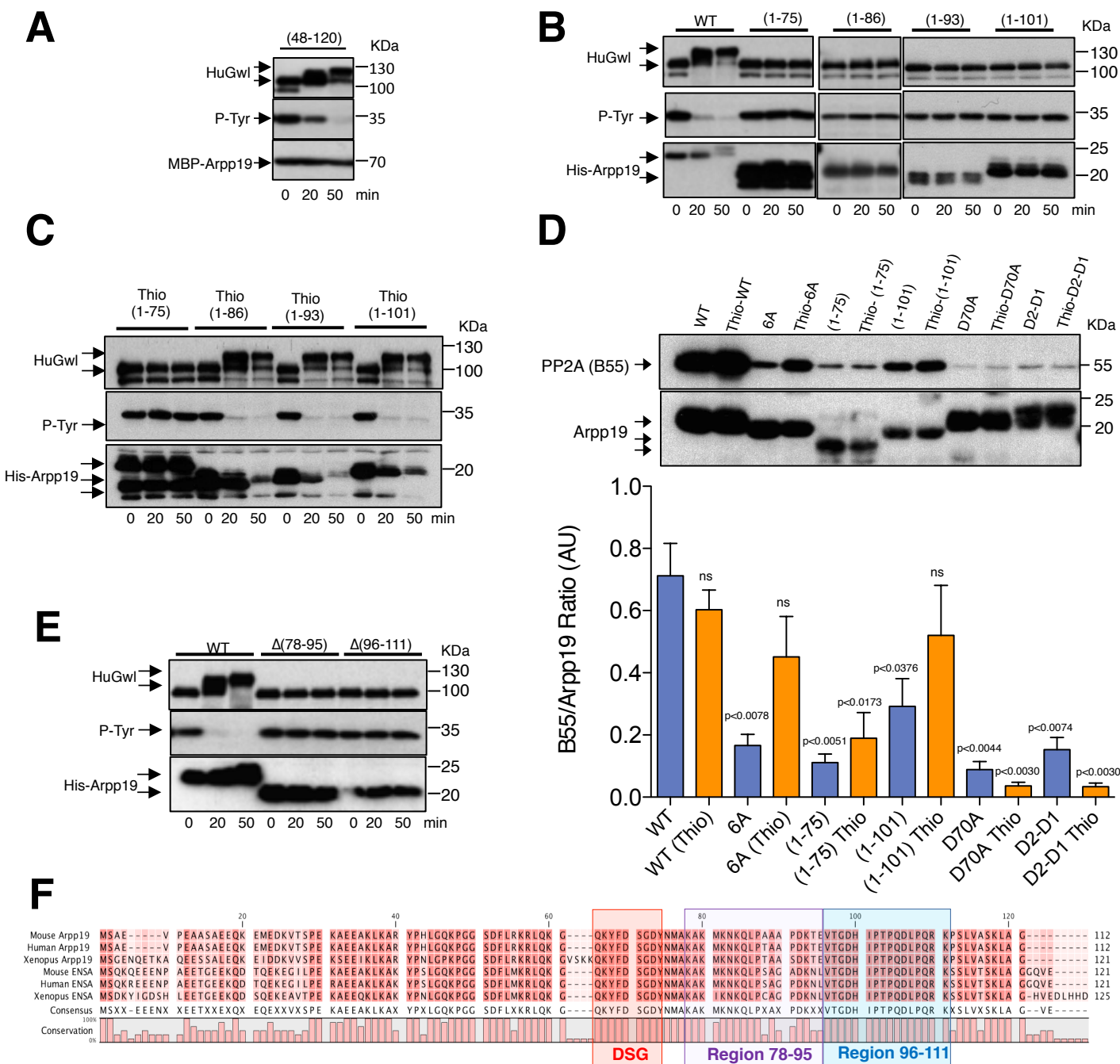


**Supplementary Figure 2.** Aromatic and acidic residues flanking S71 Gwl site of Arpp19 are essential to promote mitotic entry to Arpp19 devoid extracts.

**(A)** The wildtype or the indicated mutants of Arpp19 were supplemented simultaneously with a trace amount of human GwlK72M and the capacity of this protein to promote mitotic entry in Arpp19-depleted extracts analyzed. The phosphorylation of human ectopic GwlK72M together with the phosphorylation of Tyr 15 of Cdk1 and the levels of ectopic Arpp19 wildtype or mutant proteins were measured by western blot (left panels). In order to fully deplete Arpp19, four rounds of immunoprecipitation were performed in CSF extracts. The amount of Arpp19 remaining upon four immunoprecipitations is shown (right panel). IgG: G Immunoglobulines. SN IP: Supernatant of the fourth immunoprecipitation.

**(B)** As for (B) except that the wildtype or the mutant forms Arpp19 were thio-phosphorylated “*in vitro*” by GwlK72M before being added to the Arpp19-depleted extracts.

Experiments supporting the data of this figure were performed at least three times.



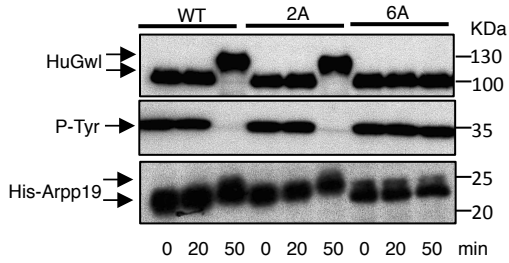
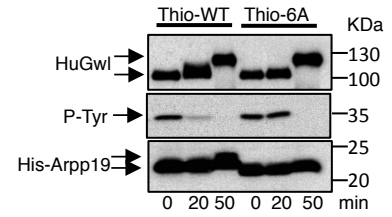
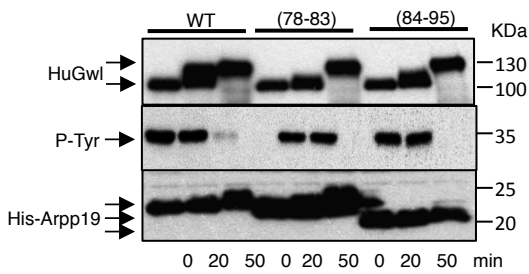
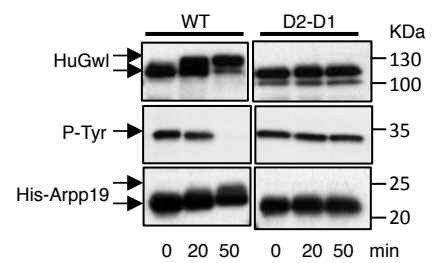
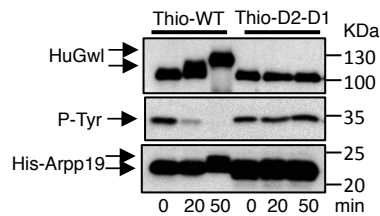
**Supplementary Figure 3.** Arpp19 PP2A-B55 inhibitory activity depends on a specific sequence of the “cassette” motif as well as on a minimal distance between this sequence and the DSG motif.

**(A)** The capacity of the (48-120) Arpp19 mutant to restore mitosis in Arpp19-depleted extracts is determined.

**(B), (C)** and **(E)** as for (A) except that the indicated mutants were thio-phosphorylated or not as indicated and tested.

**(D)** The wildtype and the indicated Arpp19 mutants were thio-phosphorylated or not and submitted to His-pulldown as indicated in material and methods except for the use of a less stringent washing of beads with XB buffer. Assays were repeated three times, B55 bound to the beads was measured and corrected by the levels of Arpp19. Represented is the mean B55/Arpp19 ratio +/- SD. Two-tailed unpaired Student t tests were performed in each pulldown to determine statistical relevance. p versus wildtype Arpp19. ns: non-significant.

**(F)** Depicted are sequence homology of Arpp19 between different species. Regions 78-95 and 96-111 as well as the DSG motif are indicated with violet, blue and red squares respectively. Note that region 96-111 is highly conserved. All the experiments were performed by triplicate.

**A****B****C****D****E**

**Supplementary Figure 4.** Arpp19 PP2A-B55 inhibitory activity depends on a specific sequence of the “cassette” motif as well as on a minimal distance between this sequence and the DSG motif.

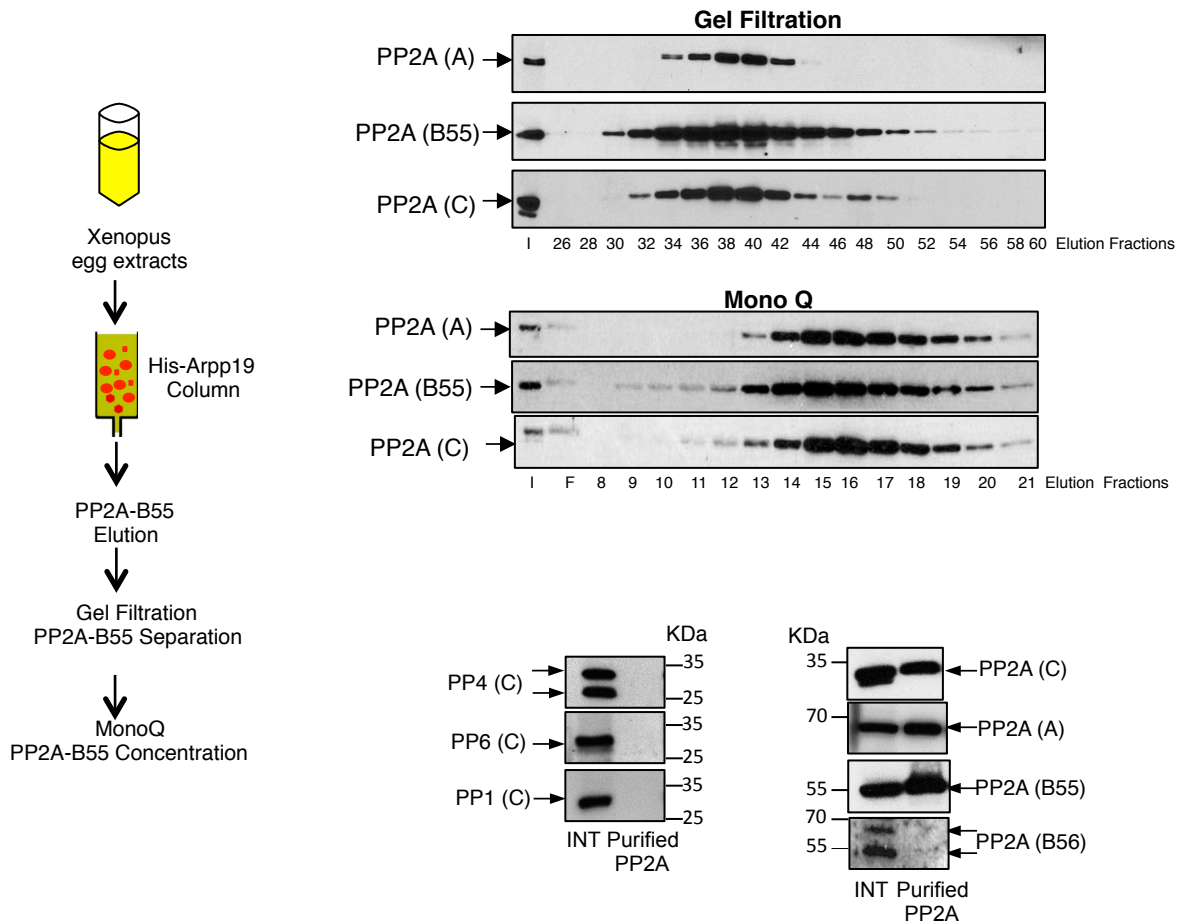
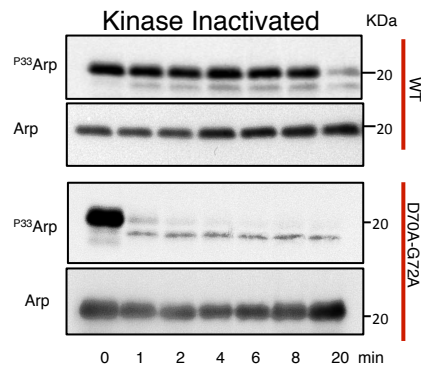
**(A)** The capacity of the 2A and 6A Arpp19 mutants to restore mitosis in Arpp19-depleted extracts is determined.

**(B)** 6A Arpp19 mutant form was thio-phosphorylated and its capacity to restore mitosis in Arpp19-depleted extracts tested.

**(C)** and **(D)** as for (A) except that the indicated mutants were tested.

**(E)** Rescue assays were performed as for the other mutants except that a thio-D2-D1 phosphorylated form was used.

Experiments supporting the data of this figure were performed at least three times.

**A****B**

**Supplementary Figure 5.** Schematic representation of the procedure used to purify PP2A-B55 phosphatase from Xenopus egg extracts.

**(A)** Shown are the amount of PP2A A, B55 and C subunits present in the elution fractions of gel filtration and MonoQ columns. The levels of PP4, PP6, PP1, and A, C, B55 and B56 subunits of PP2A were also examined in the final mix of fractions 15, 16 and 17 representing the pick of purified PP2A-B55 used for the rest of the study. I: Interphase extracts; F: flow through.

**(B)** The dephosphorylation of S67/S71 of the wildtype or the D70A-G72A double mutant of Arpp19 were assayed in kinase-inactivated extracts and revealed by autoradiography. The amount of this Arpp19 mutant form in each sample is also shown. All the experiments were repeated at least three times.

| REAGENT OR RESOURCE                                 | SOURCE               | IDENTIFIER       |
|-----------------------------------------------------|----------------------|------------------|
| <b>BACTERIAL AND VIRUS STRAINS</b>                  |                      |                  |
| BL21DE3 Competent E.Coli                            | New England Biolabs  | Cat#C2527H       |
| DH5α E. Coli                                        | New England Biolabs  | Cat#C2987I       |
| <b>CHEMICALS, PEPTIDES AND RECOMBINANT PROTEINS</b> |                      |                  |
| [gamma-P33] ATP                                     | HARTMANN ANALYTIC    | Cat#SRF-301      |
| Pfu ultra II fusion DNA polymerase                  | Agilent              | Cat#600670       |
| ATPγS                                               | Sigma                | Cat#A1388        |
| TALON Superflow Metal Affinity Resin                | Takara               | Cat#635506       |
| PVDF transfer membrane                              | Millipore            | Cat#88518        |
| Protan Nitrocellulose membran                       | Amersham             | Cat#GE10600016   |
| Dynabeads protein G                                 | Life Technologies    | Cat#10004D       |
| Histone H1                                          | Sigma                | Cat#14_155       |
| BSA                                                 | Sigma                | Cat#A7906        |
| His-PureTMNiNTA magnetic beads                      | Life Technologies    | Cat#88832        |
| CNBr-activated sepharose 4B                         | GE Healthcare        | Cat#17_0430_01   |
| Amylose Resin High Flow                             | Biolabs              | Cat#E80225       |
| Sulfo-MBS                                           | Thermo Scientific    | Cat#22312        |
| HiLoadTM16/600 Superdex                             | Thermo Scientific    | Cat#28_9893_35   |
| MonoQ 5/50 GL                                       | GE-Healthcare        | Cat#GE17_5166_01 |
| Recombinant GST-Human Greatwall K72M mutant         | Vigneron et al. 2011 | N/A              |
| GST-Xenopus B56 gamma                               | This study           | N/A              |

| REAGENT OR RESOURCE                                        | SOURCE     | IDENTIFIER |
|------------------------------------------------------------|------------|------------|
| <b>OLIGONUCLEOTIDES</b>                                    |            |            |
| Forward and reverse primers for Arpp19 K36A/K38A/R40A:     | Eurogentec | This Study |
| 5'<br>GAAGTCAGAGGAGATAGCGTTAGCAG<br>CAGCGTATCCTAACCTCGG 3' |            |            |
| 5'<br>CCGAGGTTAGGATACGCTGCTGCTAA<br>CGCTATCTCCTCTGACTTC 3' |            |            |
| Forward and reverse primers for Arpp19 Q66A:               | Eurogentec | This Study |
| 5'GGCGTAAGTAAAAAGGCAAAATATT<br>TTGACTCTGGG 3'              |            |            |
| 5'<br>CCCAGAGTCAAAATATTTTGCCTTTTTA<br>CTTACGCC 3'          |            |            |
| Forward and reverse primers for Arpp19 K67A:               | Eurogentec | This Study |
| 5'<br>GGCGTAAGTAAAAAGCAAGCATATTT<br>TGACTCTGGGGAC 3'       |            |            |
| 5'<br>GTCCCCAGAGTCAAAATATGCTTGCTT<br>TTTACTTACGCC 3'       |            |            |
| Forward and reverse primers for Arpp19 Y68A:               | Eurogentec | This Study |
| 5'<br>GTAAGTAAAAAGCAAAAAGCTTTTGA<br>CTCTGGGGAC 3'          |            |            |
| 5'<br>GTCCCCAGAGTCAAAAGCTTTTGTCTT<br>TTTACTTAC 3'          |            |            |
| Forward and reverse primers for Arpp19 F69A:               | Eurogentec | This Study |
| 5'<br>GTAAGTAAAAAGCAAAAATATGCTGA<br>CTCTGGGGACTACAT 3'     |            |            |
| 5'<br>ATTGTAGTCCCCAGAGTCAGCATATTT<br>TTGCTTTTACTTC 3'      |            |            |
| Forward and reverse primers for Arpp19 D70A:               | Eurogentec | This Study |
| 5'<br>AAAGCAAAAATATTTTgccTCTGGGGA<br>CTACAATATG 3'         |            |            |
| 5'<br>CATATTGTAGTCCCCAGAggcAAAATA<br>TTTTTGCTTT 3'         |            |            |

| REAGENT OR RESOURCE                                          | SOURCE     | IDENTIFIER |
|--------------------------------------------------------------|------------|------------|
| <b>OLIGONUCLEOTIDES</b>                                      |            |            |
| Forward and reverse primers for Arpp19 S71T:                 | Eurogentec | This Study |
| 5'<br>GGCCAAAAATATTTTGACACTGGGGA<br>CTACAATATGGC 3'          |            |            |
| 5'<br>GCCATATTGTAGTCCCCAGTGTCAAAA<br>TATTTTGGCC 3'           |            |            |
| Forward and reverse primers for Arpp19 G72A:                 | Eurogentec | This Study |
| 5'<br>CAAAAATATTTTGACTCTGCGGACTAC<br>AATATGGCTAAA 3'         |            |            |
| 5'<br>TTTAGCCATATTGTAGTCCGCAGAGTC<br>AAAATATTTTG 3'          |            |            |
| Forward and reverse primers for Arpp19 D73A:                 | Eurogentec | This Study |
| 5'<br>TATTTTGACTCTGGGGCCTACAATATG<br>GCTAAA 3'               |            |            |
| 5'<br>TTTAGCCATATTGTAGGCCCCAGAGTC<br>AAAATAG 3'              |            |            |
| Forward and reverse primers for Arpp19 Y74A:                 | Eurogentec | This Study |
| 5'<br>TATTTTGACTCTGGGGACGCCAATATG<br>GCTAAAGCA 3'            |            |            |
| 5'<br>TTTAGCCATATTGTAGGCCCCAGAGTC<br>AAAATAG 3'              |            |            |
| Forward and reverse primers for Arpp19 (48-120):             | Eurogentec | This Study |
| 5'<br>CGCGGATCCAGAAAGCGACTTCAGAA<br>AGGCG 3'                 |            |            |
| 5'<br>CCCAAGCTTTCAGCCAGCCAGTTTGCT<br>TGCG 3'                 |            |            |
| Forward and reverse primers for Arpp19 (1-75):               | Eurogentec | This Study |
| 5'<br>GAC-TCT-GGG-GAC-TAC-AAT-TAG-<br>GCT-AAA-GCA-AAG-ATG 3' |            |            |
| 5'<br>CAT-CTT-TGC-TTT-AGC-CTA-ATT-<br>GTA-GTC-CCC-AGA-GTC 3' |            |            |

| REAGENT OR RESOURCE                                                                                                                                                        | SOURCE     | IDENTIFIER |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|------------|
| <b>OLIGONUCLEOTIDES</b>                                                                                                                                                    |            |            |
| Forward and reverse primers for Arpp19 (1-86):<br><br>5'<br>GATGAAGAACAAGCAACTGTAAACAG<br>CTGCATCTGATAAA3'<br>5'<br>TTTATCAGATGCAGCTGTTTACAGTTG<br>CTTGTTCTTCATC 3'        | Eurogentec | This Study |
| Forward and reverse primers for Arpp19 (1-93):<br><br>5'<br>CAACAGCTGCATCTGATAAATAGGAG<br>GTTACGGGTGATCAT 3'<br>5'<br>ATGATCACCCGTAACCTCCTATTTATCA<br>GATGCAGCTGTTG 3'     | Eurogentec | This Study |
| Forward and reverse primers for Arpp19 (1-101):<br><br>5'<br>GTTACGGGTGATCATATTTAGACGCCA<br>CAAGACCTCCCT 3'<br>5'<br>GAGGGAGGTCTTGTGGCGTCTAAATA<br>TGATCACCCGTAAC 3'       | Eurogentec | This Study |
| Forward and reverse primers for Arpp19 D(78-95):<br><br>5'<br>CTCTGGGGACTACAATATGGCTGTTAC<br>GGGTGATCATATTCC 3'<br>5'<br>GGAATATGATCACCCGTAACAGCCAT<br>ATTGTAGTCCCCAGAG 3' | Eurogentec | This Study |
| Forward and reverse primers for Arpp19 D(96-111):<br><br>5'<br>GCTGCATCTGATAAAACGGAGCCGTC<br>TCTCGTTGCAAGCA 3'<br>5'<br>TGCTTGCAACGAGAGACGGCTCCGTTT<br>TATCAGATGCAGC 3'    | Eurogentec | This Study |
| Forward and reverse primers for Arpp19 2A:<br><br>5'<br>CGGGTGATCATATTCCTGCGGCACAA<br>GACCTCCCTCAAAG 3'<br>5'<br>CTTTGAGGGAGGTCTTGTGCCGAGG<br>AATATGATCACCCG 3'            | Eurogentec | This Study |

| REAGENT OR RESOURCE                                        | SOURCE     | IDENTIFIER |
|------------------------------------------------------------|------------|------------|
| <b>OLIGONUCLEOTIDES</b>                                    |            |            |
| Forward and reverse primers for Arpp19 6A:                 | Eurogentec | This Study |
| 5'<br>CATATTCCTGCGGCACAAGCCGCCGCT<br>GCAAGGAAACCGTCTCTC 3' |            |            |
| 5'<br>GAGAGACGGTTTCCTTGCAGCGGCGG<br>CTTGTGCCGAGGAATATG 3'  |            |            |
| Forward and reverse primers for Arpp19 D(78-83):           | Eurogentec | This Study |
| 5'<br>TCTGGGGACTACAATATGGCTCAACTG<br>CCAACAGCTGCATCT 3'    |            |            |
| 5'<br>AGATGCAGCTGTTGGCAGTTGAGCCA<br>TATTGTAGTCCCCAGA 3'    |            |            |
| Forward and reverse primers for Arpp19 D(84-95):           | Eurogentec | This Study |
| 5'<br>GGCTAAAGCAAAGATGAAGAACGTTA<br>CGGGTGATCATATTCC 3'    |            |            |
| 5'<br>GGAATATGATCACCCGTAACGTTCTTC<br>ATCTTTGCTTTAGCC 3'    |            |            |
| Forward and reverse primers for Arpp19 (D2-D1):            | Eurogentec | This Study |
| 5'<br>GGTCGACAATGTCTGGAGAAAATCAG<br>GAG 3'                 |            |            |
| 5' CGCGGATCCTCAGCCTTTAGCCATA<br>3'                         |            |            |
| Forward and reverse primers for Arpp19 S113D:              | Eurogentec | This Study |
| 5'CTCCCTCAAAGGAAACCGGATCTCG<br>TTGCAAGCAAAGTGG 3'          |            |            |
| 5'<br>CCAGTTTGCTTGCAACGAGATCCGGTT<br>TCCTTTGAGGGAG 3'      |            |            |

| REAGENT OR RESOURCE             | SOURCE                                        | IDENTIFIER |
|---------------------------------|-----------------------------------------------|------------|
| <b>RECOMBINANT DNA</b>          |                                               |            |
| Lorca et al. 1993               | Lorca et al. 1993                             | N/A        |
| pET7F1-human Cyclin A           | Generous gift of G Draetta.; Lorca et al.1992 | N/A        |
| pET15-6His-Xenopus Arpp19       | Ma et al. 2016                                | N/A        |
| pFastBac-GST-hGwlK72M           | Vigneron et al. 2011                          | N/A        |
| pET15-6His-Xenopus ENSA         | This Study                                    | N/A        |
| pMalCX2-His-Arpp19              | This Study                                    | N/A        |
| pET15b-human PKA                | This study                                    | N/A        |
| pCMVsport6–Xenopus B56 gamma 3’ | This study                                    | N/A        |
| pET15b-human PRC1               | This Study                                    | N/A        |

REFERENCES:

Lorca, T. *et al.* Cyclin A-cdc2 kinase does not trigger but delays cyclin degradation in interphase extracts of amphibian eggs. *Journal of cell science* **102 ( Pt 1)**, 55–62 (1992).

Lorca, T. *et al.* Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. *Nature* **366**, 270–3 (1993).

Vigneron, S. *et al.* Characterization of the Mechanisms Controlling Greatwall Activity. *Molecular and Cellular Biology* **31**, 2262–2275 (2011).

Ma, S. *et al.* Greatwall dephosphorylation and inactivation upon mitotic exit is triggered by PP1. *Journal of cell science* **129**, 1329–39 (2016).