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Supplemental Data

An Isoform-Selective, Small-Molecule Inhibitor Targets the Autoregulatory Mechanism of p21-Activated Kinase

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Supplemental Experimental Procedures

Synthesis of IPA-3 (2,2'-Dihydroxy-1,1'-dinapthyldisulfide) and 1-mercapto-2-hydroxynapthalene. All chemicals and reagents were used as received from the supplier. Sodium thiocyanate, 2-naphthol, and zinc were obtained from Aldrich. Bromine, sulfuric acid, hydrochloric acid and sodium hydroxide were obtained from Fisher. Glacial acetic acid was obtained from Mallinckrodt. TLC analysis was performed on EMD Silica Gel 60 F_{254} coated plates. Melting point analysis was determined using a Fisher-Johns apparatus. ^{1}H-NMR analysis was recorded on a Bruker Advance WB 300MHz instrument with TMS as an internal standard. Mass Spectroscopy was carried out at HT Laboratories (San Diego, CA) using electrospray.

2,2'-Dihydroxy-1,1'-dinapthyldisulfide (2) Glacial acetic acid (325 ml), sodium thiocyanate (36 g, 0.444 mol) and 2-napthol (16.8 g, 0.117 mol) were stirred with cooling in an ice bath. Then, bromine (6 ml, 18.71g , 0.117mol) in 60 ml of glacial acetic acid was added dropwise over 30 min. A precipitate formed and the reaction was stirred for an additional 30 min at room temperature. Next, the mixture was poured into one liter of

water and the precipitate was collected, washed with water and dried under reduced pressure. This material, hydroxynapthyl-1-thiocyanate (**1**), was dissolved in 250 ml of water containing 25 g of sodium hydroxide and was heated to 95-100^{o}C for 30 min. After cooling, the pH of the solution was adjusted to 6.0-6.5 with 6 N H_2SO_4. The resulting precipitate was filtered and dried under reduced pressure. The solid was recrystallized from acetic acid, washed with water and dried to yield 9.13 g (44%) of disulfide **2**. TLC analysis (CH_2Cl_2 9/MeOH 1) Rf = 0.96 and (pet ether 1/ether 1) Rf = 0.56. ^{1}HNMR (CD_3Cl,): 7.995 δ (d, 2H, 4,4'), 7.792 δ (d, 2H, 5,5'), 7.720 δ (d, 2H, 8,8'), 7.374-7.319 δ (m, 4H, 6,6, & 7,7'), 7.03 δ (d, 2H, 3,3'), 6.587 δ (s, 2H, OH). Mp 171.5-172.5 ^{o} C (lit 168^{o}C). ESIMS: calculate 350.04 for $C_{20}H_{14}O_2S_2$; found $[M-H]^-$ = 349.

1-Mercapto-2-hydroxynapthalene (IPA-3R) (3) 2.5 g (7.1 mmol) of 2,2'-dihydroxy-1,1'-dinapthyldisulfide (2), 2.0 g (31 mmol) of powdered zinc, and 25 ml of acetic acid were heated in an oil bath to 96^{o}C. Concentrated hydrochloric acid (5 ml) was added to dissolve any salts that formed during the reaction. After cooling, the reaction mixture was poured with rapid stirring, into 250 ml water. The product was collected by filtration, washed with water, and dried. The crude thiol was recrystallized twice from petroleum ether to give 0.822 g (33% yield) of (**3**) as pale yellow needles. TLC (silica gel) $CHCl_3$ 9/MeOH 1/AcOH 3: Rf = 0.93 and EtOAc 3/Hexane 7: Rf = 0.54. The melting point was 52-53^{o}C (literature 55^{o}C) ^{1}HNMR ($CDCl_3$): 8.340 δ (d, 1H, 4), 7.920 δ (d-2H, 5,8), 7.607 δ (t, 1H, 7), 7.381 δ (t, 1H, 6), 7.249 δ (d, 1H, 3), 6.878 δ (s, 1H, OH), 2.757 δ (s, 1H, SH). ESIMS calculated 176.03 for $C_{10}H_8OS$; found $[M-H]^-$ = 175.

DNA Mutagenesis. Pak1-CS double mutant (cysteine 360 to serine and cysteine 411 to serine) was generated in two sequential steps by PCR from histidine-tagged full-length human Pak1 [1]. The following primer pairs were used in each step (mutated nucleotides in bold): 360-forward: 5'-G GTG ACA GAA ACT T**C**C ATG GAT GAA GGC C-3', 360-reverse: 5'-G GCC TTC ATC CAT G**G**A AGT TTC TGT CAC C-3', 411-forward: 5'-G CTA ACT GAC TTT GGA TTC T**C**T GCA CAG ATA ACC CCA GAG C-3', 411-reverse: 5'-G CTC TGG GGT TAT CTG TGC A**G**A GAA TCC AAA GTC AGT TAG C-3'. Pak5 ATP binding pocket mutant (Pak5-AM) was generated by PCR mutagenesis of methionine 523 to encode glycine using the template pNIC28-Bsa4 coding for the kinase domain of Pak5 [2]. Primers used were (mutated nucleotides in bold): 523-forward: 5'- G CTC TGG GTG GTC **GG**G GAG TT**C** CTA GAA GGT GGT GCC-3', 523-reverse: 5'- GGC ACC ACC TTC TAG **G**AA CTC C**C**C GAC CAC CCA GAG C-3'. An additional silent mutation was incorporated into the primers to eliminate an XbaI site to facilitate mutation detection.

Supplemental References

1. Rennefahrt, U.E., Deacon, S.W., Parker, S.A., Devarajan, K., Beeser, A., Chernoff, J., Knapp, S., Turk, B.E., and Peterson, J.R. (2007). Specificity profiling of Pak kinases allows identification of novel phosphorylation sites. J Biol Chem *282*, 15667-15678.
2. Eswaran, J., Lee, W.H., Debreczeni, J.E., Filippakopoulos, P., Turnbull, A., Fedorov, O., Deacon, S.W., Peterson, J.R., and Knapp, S. (2007). Crystal Structures of the p21-activated kinases PAK4, PAK5, and PAK6 reveal catalytic domain plasticity of active group II PAKs. Structure *15*, 201-213.

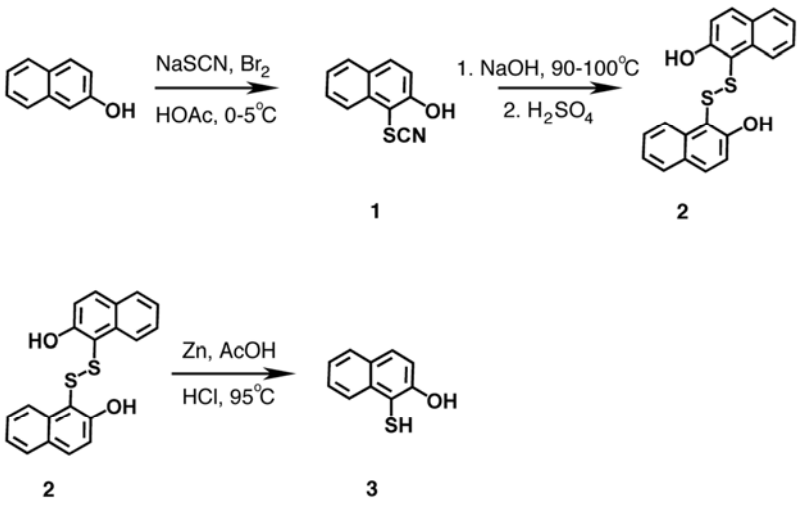

Figure S1. Synthesis Scheme for IPA-3 (2,2'-Dihydroxy-1,1'-dinapthyldisulfide) and IPA-3R (1-mercapto-2-hydroxynapthalene)

Details of the synthesis and compound characterization are presented in Supplemental Experimental Procedures

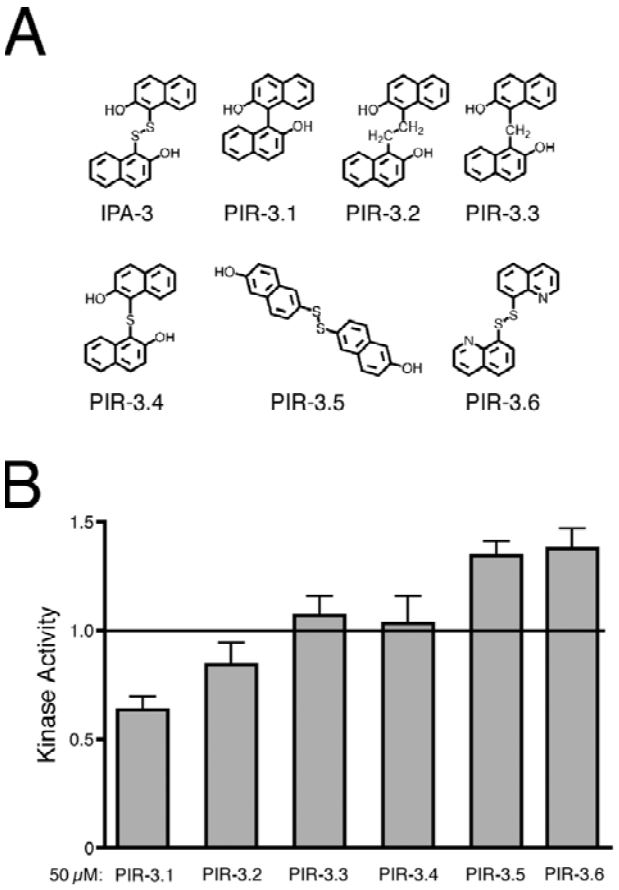

Figure S2. Inhibitory Activity of IPA-3 Structural Relatives

(A) Structure of IPA-3 and Pak1 inhibitor relatives (PIR). (B) Inhibitory activity of IPA-3 structural relatives. Pak1 was pre-incubated with 50 μM of the indicated compound. Kinase reactions were initiated by the addition of Cdc42-GTPγS, MBP, and a mixture of 1 mM ATP and [^{32}P]-γ-ATP. Samples were analyzed either by SDS-PAGE/PhosphorImager analysis or by precipitation on P81 filter paper and scintillation counting. Kinase activity is reported as phosphate incorporation onto MBP expressed as a ratio to MBP phosphorylation in control reactions in the presence of solvent alone (1% DMSO) and Cdc42. Error bars indicate the standard error of the mean. n = 5.

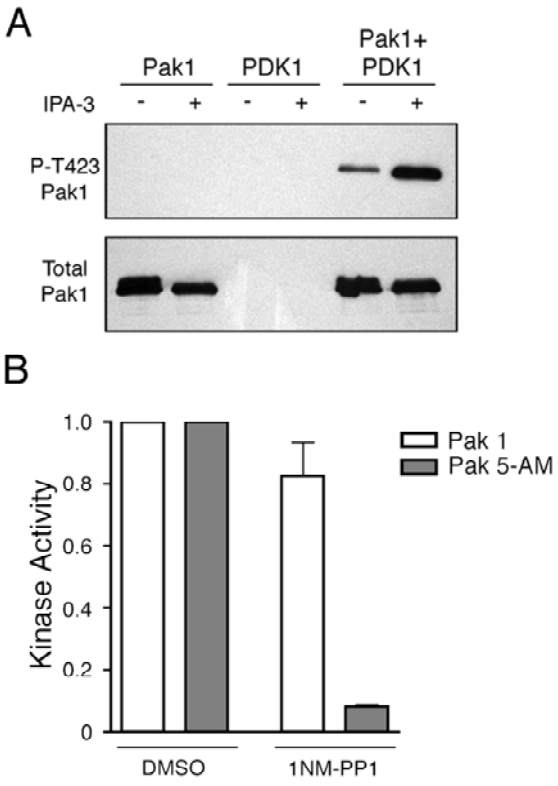

Figure S3. PDK1 Dependent Phosphorylation of Pak1 and 1NM-PP1 Kinase Selectivity

(A) IPA-3 promotes the accessibility of the Thr423 to phosphorylation by PDK1. Pak1 was incubated with 1% DMSO or IPA-3 followed by addition of full length human PDK1 (#7386, Cell Signaling technologies) and 50 μM ATP. Reactions were analyzed using phospho-specific antibodies against Pak1 Thr423 or total Pak1. Data is representative of three experiments. (B) Selective inhibition by 1NM-PP1 Pak1 or Pak5-AM were incubated with DMSO or 2 μM 1NM-PP1 followed by addition of 125 μM sphingosine, MBP and a mixture of 50 μM ATP and [^{32}P]-γ-ATP for 10 min at 30 °C. Samples were analyzed by SDS-PAGE followed by Phosphoimager analysis. Kinase activity is reported as phosphate incorporation into MBP expressed as a ratio to MBP phosphate

incorporation in the reaction containing kinase and solvent alone (1% DMSO). Error bars indicate the standard error of the mean. n = 3.

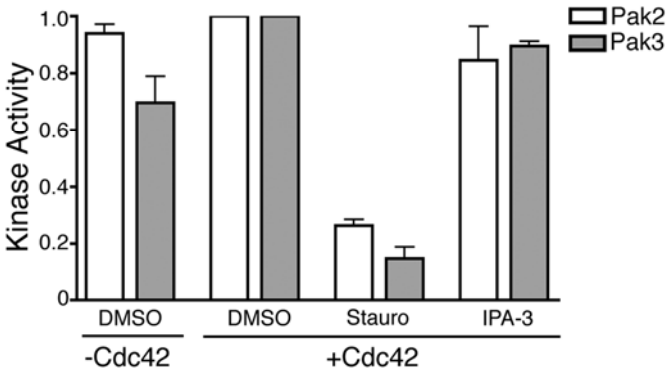

Figure S4. Inhibitory Activity of IPA-3 towards Z'-Lyte™ Kinases Pak2 and Pak3

Full-length Pak 2 or 3 proteins used in the Z'-Lyte™ kinase profiling screen were obtained from Invitrogen. Kinases were pre-incubated with 10 μM of the indicated compound or solvent control (1% DMSO). Kinase reactions were started by the addition of buffer or Cdc42-GTPγS, MBP, 1mM ATP and [^{32}P]-γ-ATP. Samples were analyzed by scintillation counting as described in Figure 2. Kinase activity is reported as phosphate incorporation onto MBP expressed as a ratio to MBP phosphorylation in control reactions in the presence of solvent alone (1% DMSO). Error bars indicate the standard error of the mean. n = 3. The lack of activation of the Z'-Lyte™Pak2 and Pak3 by Cdc42- GTPγS, unlike what is observed for purified Pak2 and Pak3 prepared in our laboratory (Figure 5), suggests that these kinases are pre-activated as supplied by Invitrogen.

Table S1. Inhibition of Z'-Lyte™ kinases by 10 µM IPA-3

Kinase Tested	[ATP] µM	Mean Percent Inhibition	*1 mM DTT* present?	*Z' score*
ABL1	10	4.4		0.79
ABL1 E255K	10	9.3		0.75
ABL1 G250E	10	3.3		0.79
ABL1 T315I	10	10.4		0.79
ABL1 Y253F	10	14.5		0.92
ABL2 (Arg)	10	8.5		0.91
ACVR1B (ALK4)	10	1.4	*	0.80
ADRBK1 (GRK2)	10	0.1		0.89
ADRBK2 (GRK3)	10	19.1		0.77
AKT1 (PKB alpha)	10	17.4		0.93
AKT2 (PKB beta)	10	68.0		0.92
AKT3 (PKB gamma)	10	6.4		0.81
ALK	10	6.9		0.71
AURKB (Aurora B)	10	-3.3		0.67
AURKC (Aurora C)	10	3.9		0.89
BLK	10	38.8		0.92
BMX	10	8.9		0.92
BRAF	100	41.3		0.62
BRAF V599E	100	1.4		0.81
BRSK1 (SAD1)	10	43.4		0.94
BTK	10	27.4		0.83
CAMK1D (CaMKI delta)	10	26.0		0.64
CAMK2A (CaMKII alpha)	10	5.6		0.82

CAMK2B (CaMKII beta)	10	-23.2		0.80
CAMK2D (CaMKII delta)	10	8.6		0.79
CAMK4 (CaMKIV)	10	20.4		0.88
CDC42 BPA (MRCKA)	10	-4.0		0.86
CDC42 BPB (MRCKB)	10	-2.4		0.90
CDK1/cyclin B	10	7.7		0.83
CDK2/cyclin A	10	-5.7		0.53
CDK5/p35	10	5.0		0.53
CHEK1 (CHK1)	10	-4.3		0.64
CHEK2 (CHK2)	10	4.0		0.54
CLK1	100	24.6		0.89
CLK2	10	11.8		0.75
CLK3	100	6.9		0.88
CSF1R (FMS)	10	0.3		0.89
CSK	10	2.2		0.90
CSNK1A1 (CK1 alpha 1)	10	-7.5	*	0.53
CSNK1D (CK1 delta)	10	8.8		0.87
CSNK1E (CK1 epsilon)	10	0.5		0.73
CSNK1G1 (CK1 gamma 1)	10	-4.0		0.82
CSNK1G2 (CK1 gamma 2)	10	29.1		0.66
CSNK1G3 (CK1 gamma 3)	10	28.9		0.87
CSNK2A1 (CK2 alpha 1)	10	7.4		0.81
CSNK2A2 (CK2 alpha 2)	10	-1.8		0.86
DAPK3 (ZIPK)	10	-0.3		0.86
DCAMKL2 (DCK2)	100	0.8		0.88
DYRK1A	10	2.1		0.89
DYRK3	10	22.9		0.80
DYRK4	10	-1.7		0.92
EGFR (ErbB1)	10	8.3	*	0.68

EGFR L858R (ErbB1 L858R)	10	10.5	*	0.67
EGFR L861Q (ErbB1 L861Q)	10	12.9	*	0.66
EPHA1	10	23.8		0.88
EPHA2	10	-6.0		0.53
EPHA3	10	6.4		0.87
EPHA4	100	49.9		0.89
EPHA5	10	12.9		0.87
EPHA8	10	7.4		0.80
EPHB1	10	3.2		0.96
EPHB2	10	0.9		0.87
EPHB3	10	7.5		0.95
EPHB4	10	30.2		0.87
ERBB2 (HER2)	10	12.2	*	0.74
ERBB4 (HER4)	10	16.5	*	0.72
FER	10	19.1		0.92
FES (FPS)	10	9.4		0.82
FGFR1	10	17.3	*	0.81
FGFR2	10	13.2	*	0.80
FGFR3	10	10.7	*	0.71
FGFR3 K650E	10	19.3	*	0.77
FGFR4	10	13.0	*	0.74
FGR	10	51.1		0.93
FLT1 (VEGFR1)	100	11.1	*	0.80
FLT3	10	7.2		0.86
FLT3 D835Y	10	0.2		0.83
FLT4 (VEGFR3)	10	11.4	*	0.68
FRK (PTK5)	10	12.2		0.87

FYN	10	24.2		0.94
GRK4	10	68.7		0.82
GRK5	10	4.1	*	0.79
GRK6	10	33.1		0.80
GRK7	10	-23.5		0.64
GSK3A (GSK3 alpha)	10	66.8		0.87
GSK3B (GSK3 beta)	10	53.9		0.93
HCK	10	12.0		0.91
HIPK1 (Myak)	10	-11.4		0.87
HIPK4	10	1.1		0.88
IGF1R	10	0.6		0.84
IKBKB (IKK beta)	10	30.0		0.79
INSR	10	12.1	*	0.79
INSRR (IRR)	100	16.2	*	0.78
IRAK4	10	4.9	*	0.77
ITK	10	4.3		0.76
JAK2	10	8.5	*	0.78
JAK2 JH1 JH2	100	25.9		0.87
JAK3	10	12.2	*	0.81
KDR (VEGFR2)	10	26.9		0.81
KIT	100	6.2	*	0.92
KIT T670I	10	-7.7	*	0.89
LCK	10	13.5		0.79
LYN A	10	6.5		0.83
LYN B	10	16.5		0.95
MAP2K1 (MEK1)	100	8.1		0.74
MAP2K2 (MEK2)	100	5.7		0.85
MAP2K6 (MKK6)	100	16.9		0.83
MAP3K8 (COT)	100	-0.6		0.85

MAP3K9 (MLK1)	100	3.8		0.80
MAP4K2 (GCK)	10	5.9		0.84
MAP4K4 (HGK)	10	8.3		0.91
MAP4K5 (KHS1)	10	43.7		0.76
MAPK1 (ERK2)	10	4.4		0.70
MAPK11 (p38 beta)	100	2.7		0.84
MAPK12 (p38 gamma)	10	-1.6		0.79
MAPK13 (p38 delta)	10	2.1		0.86
MAPK14 (p38 alpha)	100	70.1		0.92
MAPK3 (ERK1)	10	4.9		0.84
MAPKAPK2	10	57.4		0.82
MAPKAPK3	10	7.9		0.81
MAPKAPK5 (PRAK)	10	5.8		0.90
MARK1 (MARK)	10	2.7		0.72
MARK2	10	3.7		0.86
MATK (HYL)	10	-9.4		0.81
MERTK (cMER)	10	7.4	*	0.91
MET (cMet)	10	15.1		0.90
MET M1250T	10	10.5		0.91
MINK1	10	33.6		0.65
MST1R (RON)	10	11.8		0.83
MST4	100	12.8		0.88
MUSK	100	11.9	*	0.60
MYLK2 (skMLCK)	100	33.9		0.80
NEK1	10	49.4		0.85
NEK2	10	-1.7		0.83
NEK4	100	-1.3	*	0.74
NTRK1 (TRKA)	100	2.8		0.89
NTRK2 (TRKB)	10	10.7	*	0.85

NTRK3 (TRKC)	10	6.2		0.90
PAK2 (PAK65)	10	3.1		0.85
PAK3	10	-4.7		0.76
PAK4	10	4.9		0.76
PAK6	10	4.7		0.81
PAK7 (KIAA1264)	10	-1.9		0.80
PASK	100	4.3		0.91
PDGFRA (PDGFR alpha)	10	6.7	*	0.73
PDGFRA D842V	10	10.0	*	0.87
PDGFRA T674I	100	13.5	*	0.79
PDGFRB (PDGFR beta)	10	11.2	*	0.76
PDK1	100	13.3		0.68
PHKG1	10	4.9		0.82
PHKG2	10	5.7		0.88
PIM1	10	7.8		0.90
PIM2	10	16.1		0.90
PKN1 (PRK1)	10	9.4		0.79
PLK1	10	14.6		0.82
PLK2	10	10.8		0.72
PLK3	10	88.1		0.63
PRKACA (PKA)	10	-9.2		0.60
PRKCA (PKC alpha)	10	11.2		0.77
PRKCB1 (PKC beta I)	10	11.8		0.77
PRKCB2 (PKC beta II)	10	30.8		0.85
PRKCD (PKC delta)	10	3.9		0.86
PRKCE (PKC epsilon)	10	12.4		0.82
PRKCG (PKC gamma)	10	10.0		0.58
PRKCH (PKC eta)	10	11.4		0.86
PRKCI (PKC iota)	10	4.7		0.87

PRKCN (PKD3)	10	25.4		0.76
PRKCQ (PKC theta)	10	15.4		0.77
PRKCZ (PKC zeta)	10	6.9		0.65
PRKD1 (PKC mu)	10	17.5		0.79
PRKD2 (PKD2)	10	42.2		0.77
PRKG1	10	-3.7		0.61
PRKG2 (PKG2)	10	20.4		0.61
PRKX	10	38.4		0.57
PTK2 (FAK)	10	11.5		0.70
PTK2B (FAK2)	10	5.1	*	0.85
PTK6 (Brk)	100	-0.3	*	0.75
RAF1 (cRAF)	100	6.1		0.81
RET	10	5.1		0.86
RET V804L	10	0.8		0.88
RET Y791F	10	12.0		0.93
ROCK1	10	4.9		0.74
ROCK2	10	3.5		0.91
ROS1	100	9.9		0.91
RPS6KA1 (RSK1)	10	15.8		0.94
RPS6KA2 (RSK3)	10	11.6		0.90
RPS6KA3 (RSK2)	10	27.4		0.83
RPS6KA4 (MSK2)	10	11.1		0.76
RPS6KA5 (MSK1)	10	13.5		0.75
RPS6KA6 (RSK4)	10	41.7		0.55
RPS6KB1 (p70S6K)	10	9.3		0.91
SGK (SGK1)	10	46.4		0.88
SGK2	10	28.6		0.82
SGKL (SGK3)	10	92.6		0.85

SRC	10	18.9		0.92
SRC N1	10	34.6		0.84
SRMS (Srm)	100	22.7		0.71
SRPK1	10	-3.7		0.85
SRPK2	10	-0.4		0.86
STK22B (TSSK2)	10	10.6		0.83
STK22D (TSSK1)	10	0.0		0.80
STK23 (MSSK1)	100	23.9		0.92
STK24 (MST3)	100	18.7		0.91
STK25 (YSK1)	10	7.1		0.92
STK3 (MST2)	10	0.4		0.85
STK4 (MST1)	10	5.0		0.90
STK6 (Aurora A)	10	41.5		0.54
SYK	10	46.9		0.91
TAOK2 (TAO1)	100	2.4		0.93
TBK1	10	7.5		0.83
TEK (Tie2)	10	22.8	*	0.73
TYRO3 (RSE)	10	31.0		0.91
YES1	10	29.1		0.88
ZAP70	10	-4.7	*	0.94