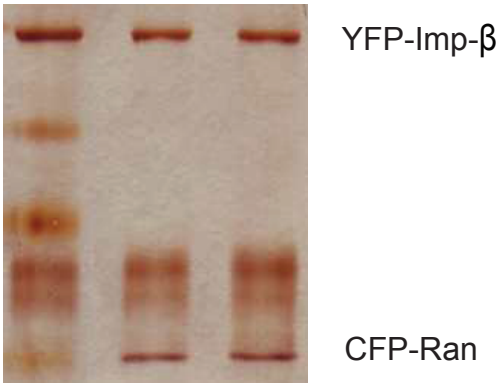
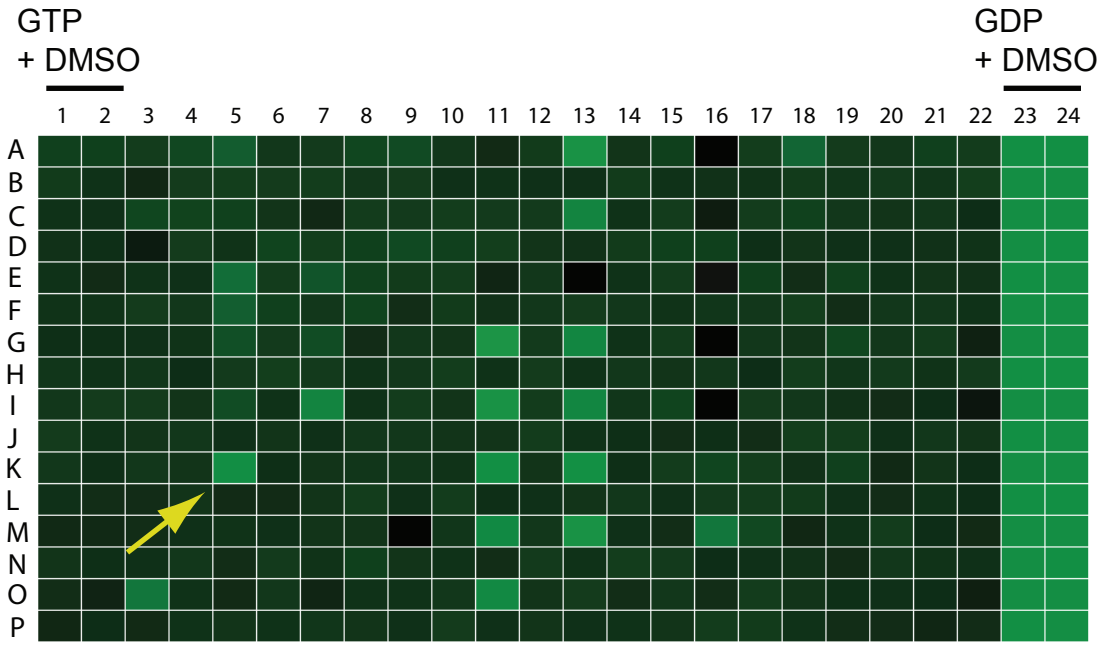
Supplemental Figure 1

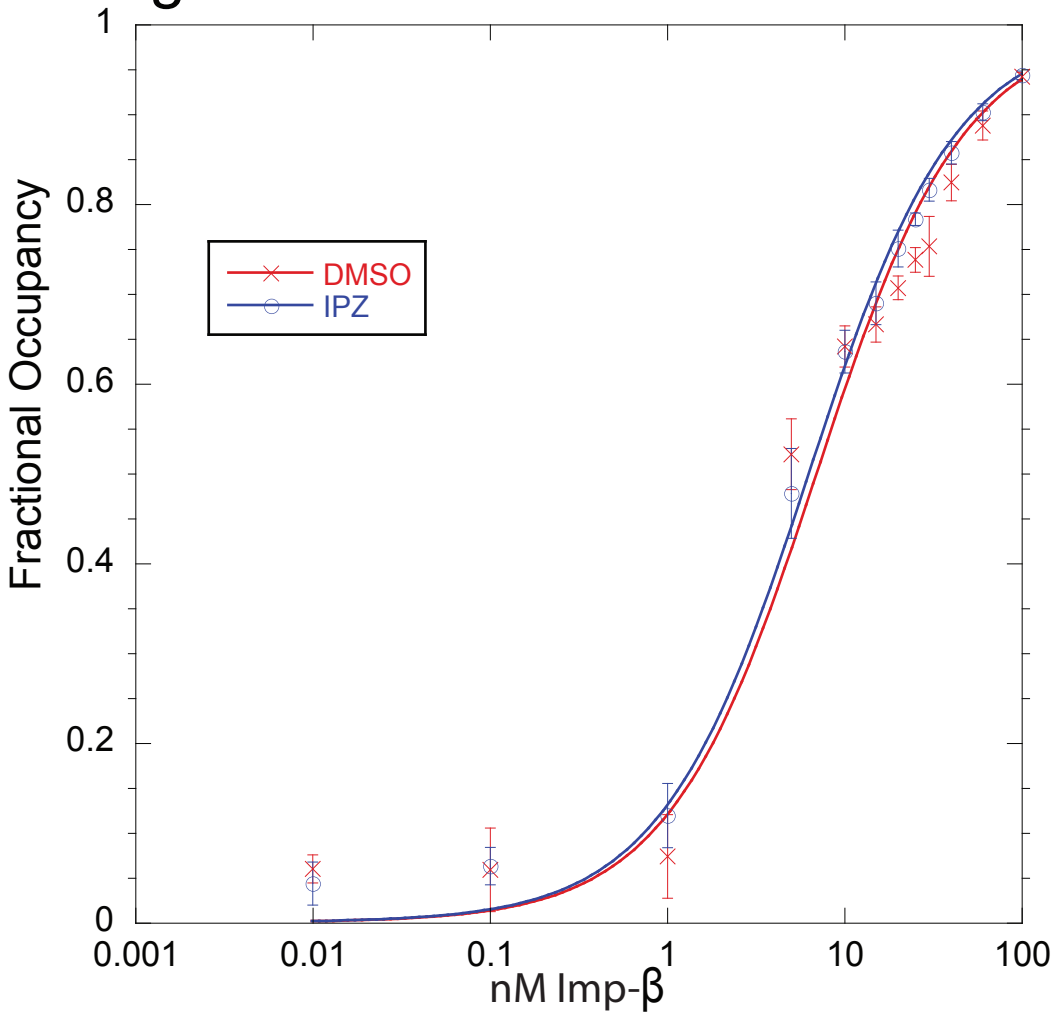


Supplemental Figure 2



Supplemental Figure 3

a



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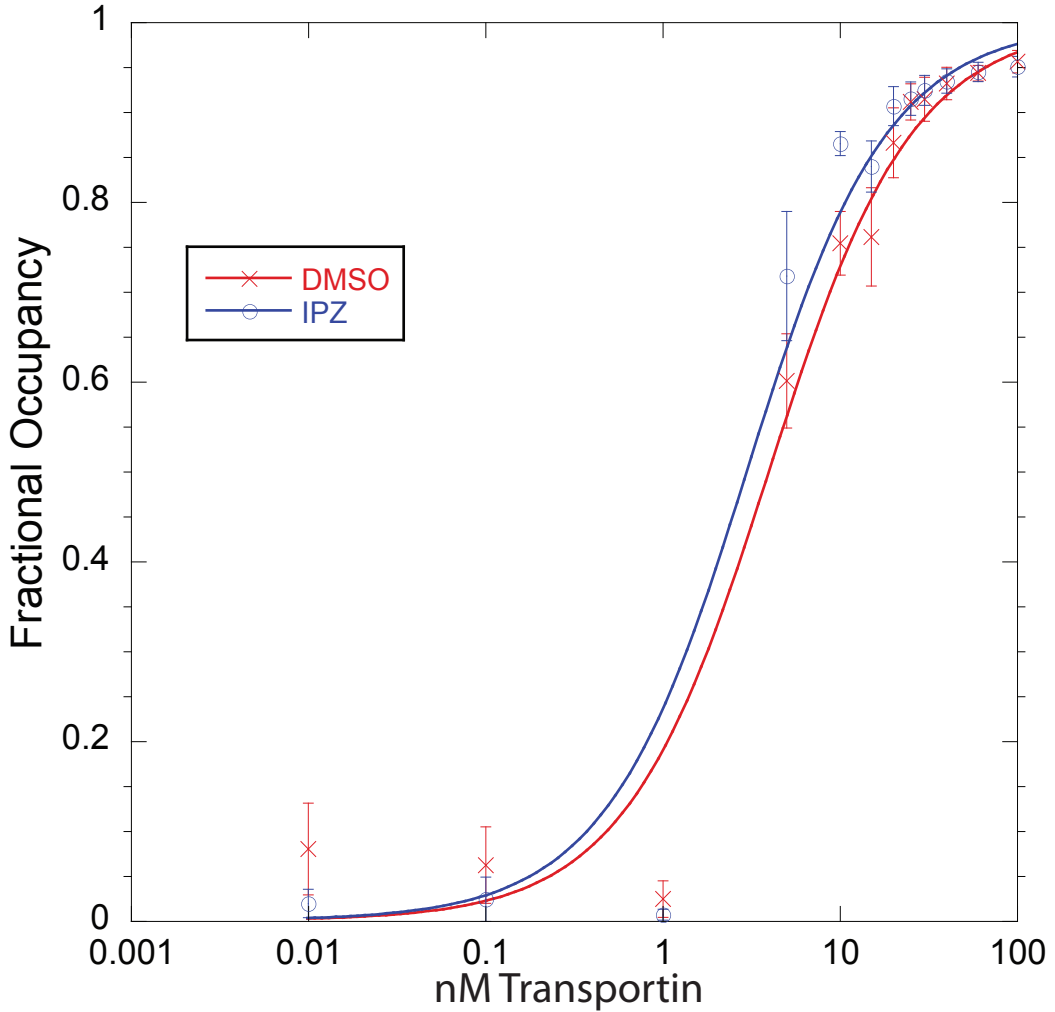
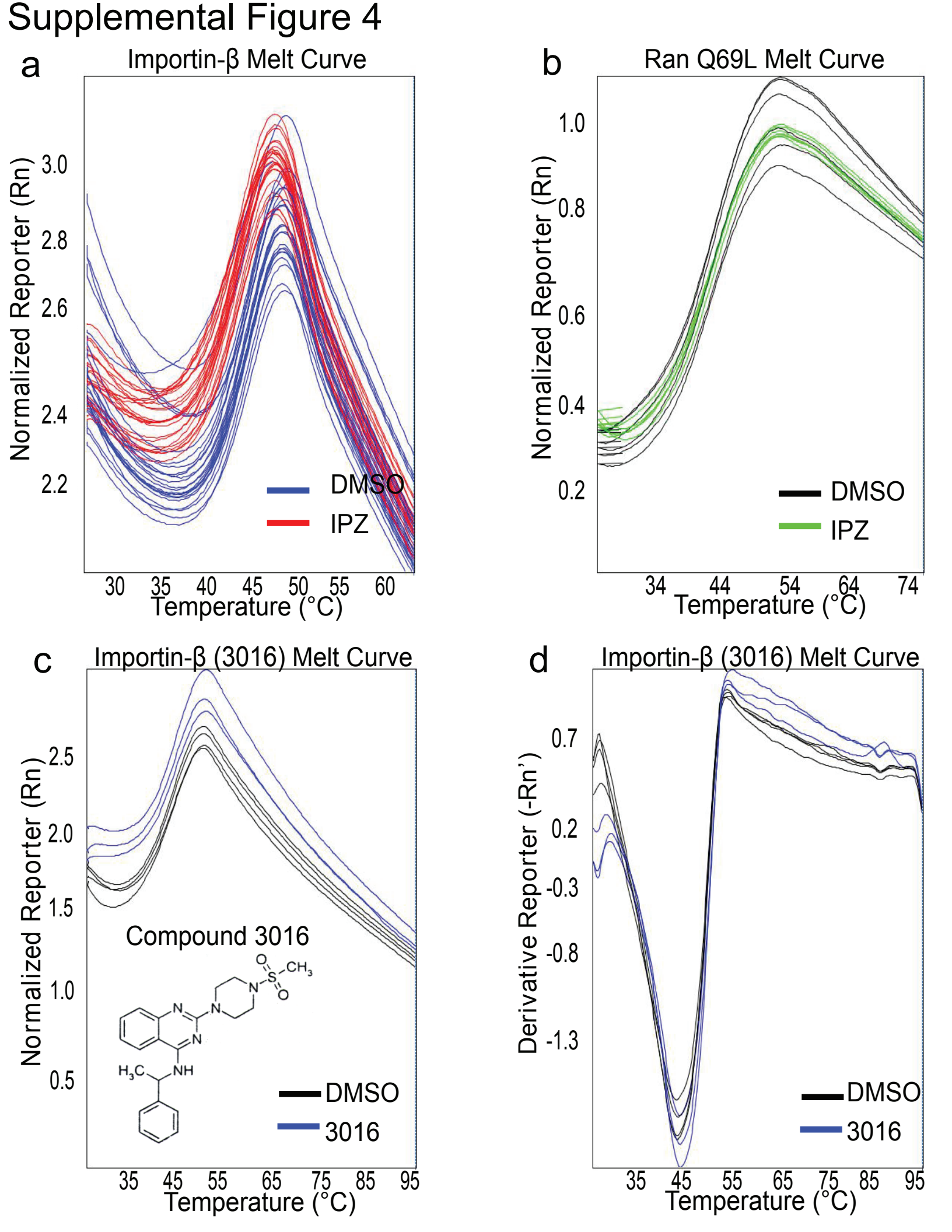
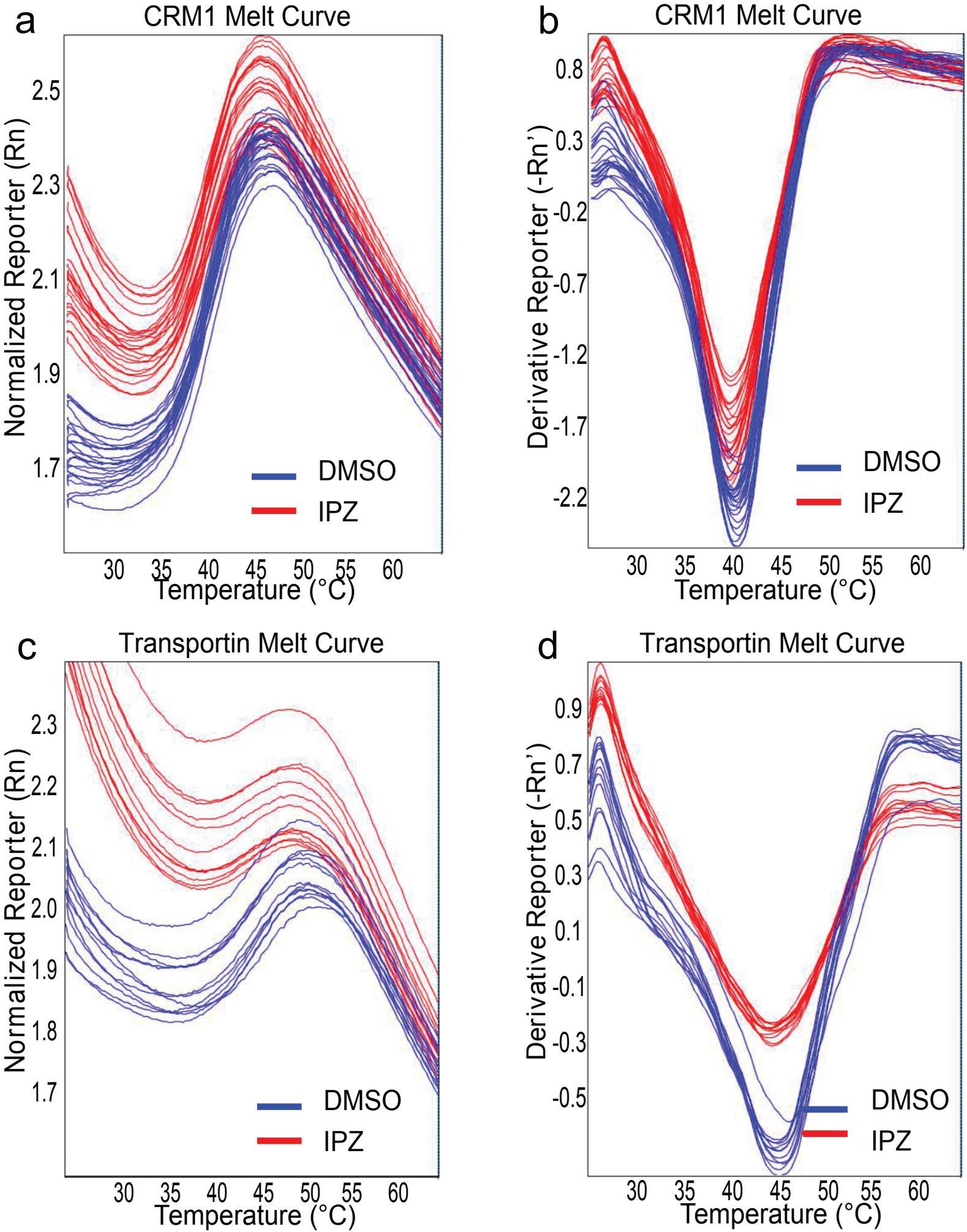


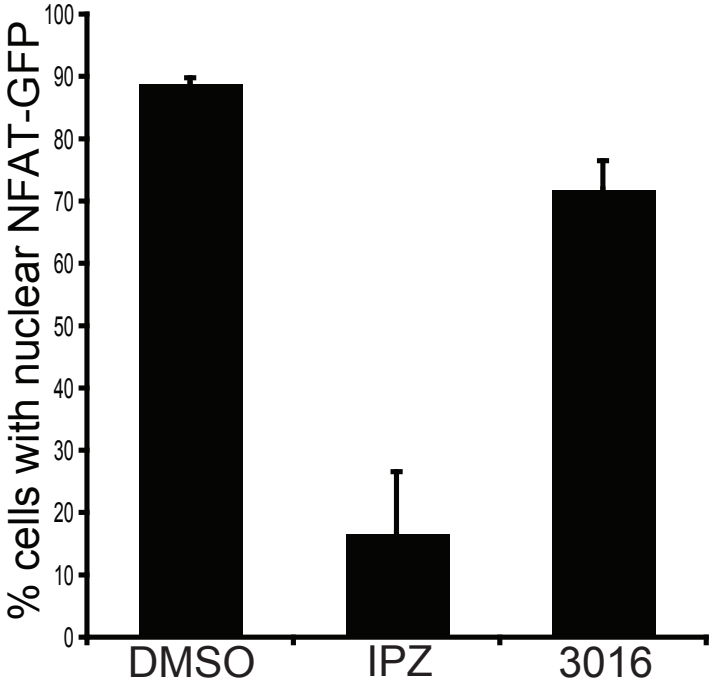
Supplemental Figure 4



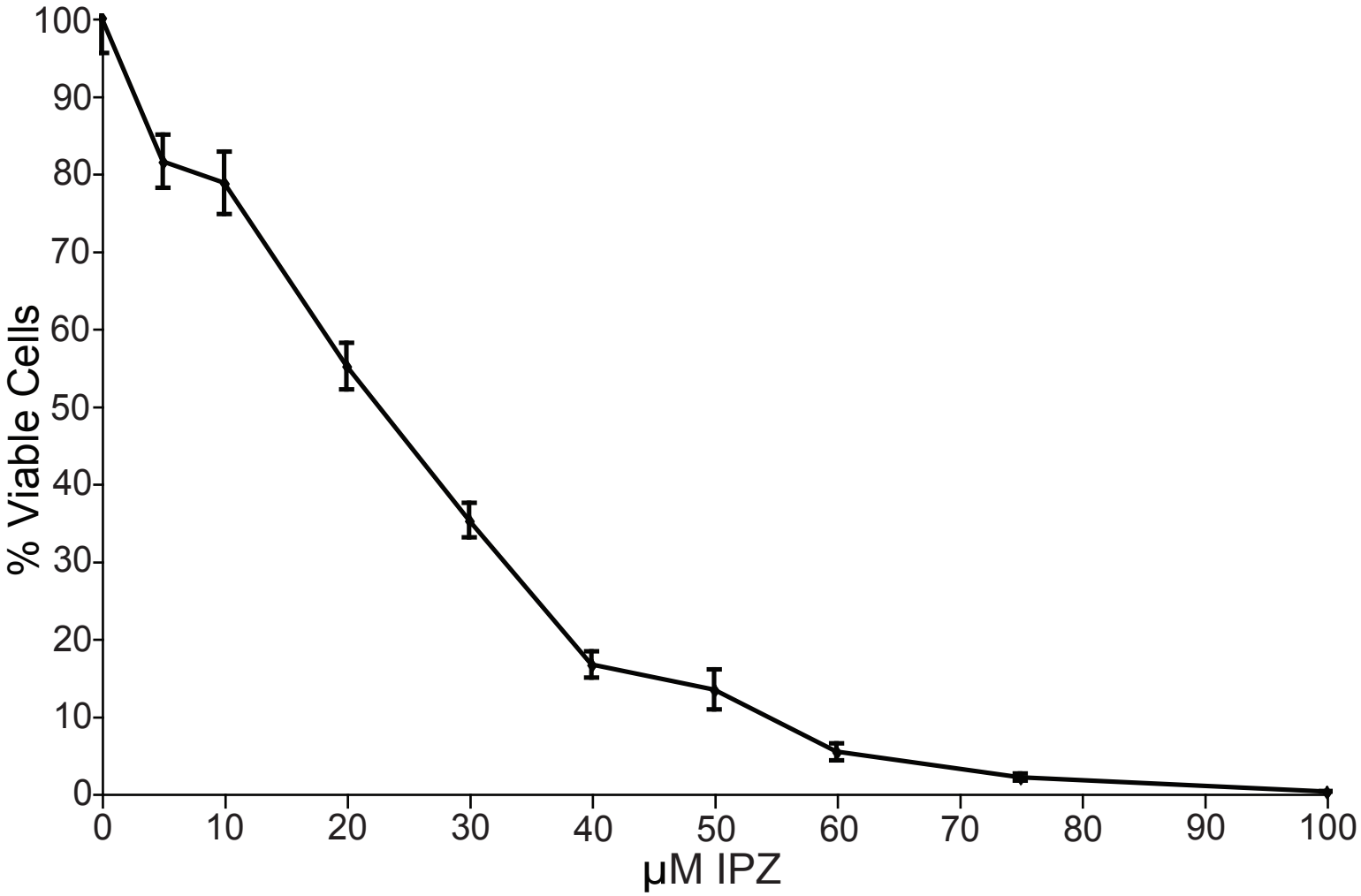
Supplemental Figure 5



Supplemental Figure 6



Supplemental Figure 7



Supplemental Figures

Supplemental Figure 1. Importazole does not affect the ability of YFP-importin-β to pull down CFP-Ran in the presence of GTP. Pull down of CFP-Ran using YFP-importin-β in the presence of GDP, GTP, or GTP plus 200 μM importazole.

Supplemental Figure 2. Heat map of a 384-well plate showing changes in FRET that monitor the interaction between CFP-Ran and YFP-importin-β. The green color represents I_{FRET}/I_{CFP}. Darker wells correspond to samples with a high I_{FRET}/I_{CFP} and lighter wells correspond to samples with a low I_{FRET}/I_{CFP}. Negative control wells occupy columns 1 and 2 (dark green). These wells include CFP-Ran, YFP- importin-β, GTP, and DMSO but no compound. Positive control wells occupy columns 23 and 24 (light green). These wells include CFP-Ran, YFP-importin-β, GDP, and DMSO but no compound. Wells in columns 3 through 22 include GTP plus compounds. The well marked with a yellow arrow showed a diminished FRET signal that was specifically due to a decrease in I_{FRET} and an increase in I_{CFP} as determined using our software, and thus was scored as a hit. Other light green wells in this plate were not scored as hits because they did not meet the criteria for a hit, which are listed in the supplemental methods section.

Supplemental Figure 3. Importazole does not destabilize the Ran/importin-β complex *in vitro*. (a) Fractional occupancy of the Ran/importin-β complex was determined via a RanGAP protection assay with increasing concentrations of importin-β in the presence of DMSO or importazole. For DMSO the K_d was estimated as 6.53 nM and for importazole the K_d was estimated as 5.86 nM. (b) Fractional occupancy of the Ran/transportin complex with increasing concentrations of transportin in the presence of DMSO or importazole. For DMSO the K_d was estimated as 3.52 nM and for importazole the K_d was estimated as 2.51 nM.

Supplemental Figure 4. Importin-β melting temperature is unaffected by the importazole related compound 3016. (a) Melting curves of 2 μM importin-β in the presence of 50 μM importazole or DMSO. (b) Melting curves of 2 μM RanQ69L in the presence of 50 μM importazole or DMSO. (c) Melting curves of 2 μM importin-β in the presence of 50 μM compound 3016 or DMSO. The structure of compound 3016 is inset in (c). (d) Negative first derivatives of importin-β melting curves in the presence of compound 3016 or DMSO where the minima represent the melting temperature (Tm).

Supplemental Figure 5. Analysis of importazole binding to transportin and CRM1 (a) Melting curves of 2 μM CRM1 (a) or 2 μM transportin (c) in the presence and absence of 50 μM importazole. Curves are the results from six experiments conducted in quadruplicate. (b, d) Negative first derivatives of CRM1 or transportin melting curves, respectively, where the minima represent the melting temperature (Tm).

Supplemental Figure 6. Compound 3016 does not block nuclear import in living cells. HEK 293 cells stably expressing GFP-NFAT were treated with DMSO, 20 μM

importazole, or 20 μM compound 3016 for 1 hour prior to a 30 min treatment with ionomycin to induce nuclear import. Results were quantified as the percentage of cells with nuclear NFAT-GFP. N=3, 100 or more cells counted under each condition. Bars represent standard error.

Supplemental Figure 7. Importazole affects HeLa cell viability. HeLa cells were treated with the carrier DMSO or varying concentrations of importazole over a period of 24 hours, and the percentage of viable cells remaining was determined using the CellTiter-Glo assay from Promega. The percentage of viable cells was normalized to the number of cells remaining following DMSO treatment alone. The IC_{50} of importazole treatment for cell viability was determined to be 22.5 μM. The curve represents the results of three independent experiments performed in quadruplicate.

Supplemental Movie 1. Importazole treatment shrinks the mitotic spindle. HeLa cells were imaged once before treatment with 50 μM importazole, and immediately after at three minute intervals for a total of 54 minutes. Each frame is a projection of five confocal slices. Scale bar = 10 μm.

Supplemental Methods

The high throughput screen. In the first step of the screen, compound dilution plates were made using a Multimek liquid handler and a Wellmate bulk dispenser by transferring 5 μl of compounds from stock plates into 384-well dilution plates (Corning, polypropylene, square wells). A bulk dispenser (Wellmate) was then used to transfer 45 μl of 2.77% DMSO (diluted with reaction buffer). This yielded a compound concentration of 100 μM and a DMSO concentration of 12.5 %. In the second step, the Multimek liquid handler was used to transfer 5 μl of solution from the dilution plates into the 384-well assay plates (Greiner, black, flat bottom). Next, the Wellmate bulk dispenser was used to transfer 20 μl of a solution containing CFP-Ran, RCC1, and GTP (diluted in reaction buffer) on top of the diluted compounds in the 384-well assay plates. This step yielded the following concentrations for each reaction component: CFP-Ran: 125 μM, RCC1: 40 nM, GTP: 400 μM, DMSO: 2.5%, and compound: 20 μM. The Wellmate bulk dispenser was then used to add 25 μl of diluted YFP-importin-β to each well in the assay plates. This step yielded the following final concentrations for each reaction component: CFP-Ran: 62.5 μM, YFP-importin-β: 62.5 μM, RCC1: 20 μM, GTP: 200 μM, DMSO: 1.25% and compound: 10 μM.
Each assay plate included 32 negative control wells (containing the GTP reaction + 1.25% DMSO) and 32 positive control wells (containing the GDP reaction). These control wells were used to set the maximum and minimum fluorescence values for each plate individually. Each compound was tested in duplicate.

In the next step the assay plates were loaded into the Analyst AD plate reader. Each well was excited with 435 nm fluorescence and emission was detected both at 475 nm (CFP) and 525 nm (YFP).

Because our high throughput screen generated a large amount of data, we designed our own software package using Perl to analyze it. Text files generated by the Analyst AD included raw fluorescence values at 475 nm and 525 nm for each well in a 384-well plate. As mentioned above, each plate included 32 negative control wells (containing the GTP mixture + 1.25% DMSO) and 32 positive control wells (containing the GDP mixture + 1.25% DMSO). Data were processed by our program in the following manner:

a. Positive control averages and standard deviations for the individual I_{CFP} and I_{FRET} emission values (475 nm and 525 nm respectively) were calculated using all 32 positive control wells. Similarly, the FRET ratio for each positive control well (I_{FRET}/I_{CFP}) was calculated and these values were used to generate an average I_{FRET}/I_{CFP} value and the standard deviation. The same calculations were performed using data from the negative control wells. Thus for each plate in the screen, our program calculated positive and negative control values that were used to set the maximum and minimum fluorescence intensities with which all other wells in the plate were compared. This allowed us to remove many fluorescent compounds that interfered with CFP or YFP fluorescence indirectly causing excessively high or low emission readings.

In the next step, fluorescence values from each well in the plate were compared to both the average positive control value and the average negative control value and their corresponding standard deviations, which were used to make error bars. Wells were removed from further consideration if:

1. I_{FRET} was greater than that of the negative control average plus three standard deviations.

2. I_{FRET} was less than that of the positive control average plus three standard deviations.

3. I_{CFP} was greater than that of the negative control average plus three standard deviations.

4. I_{CFP} was less than that of the positive control average plus three standard deviations.

5. I_{FRET}/I_{CFP} was either less than the average I_{FRET}/I_{CFP} value plus three standard deviations for the positive control wells, or greater than the average I_{FRET}/I_{CFP} value plus three standard deviations for the negative control wells.

6. I_{FRET}/I_{CFP} was not less than the average I_{FRET}/I_{CFP} value plus one standard deviation for the positive control wells. These compounds were considered to have no measurable effect on the interaction between CFP-Ran and importin-β because they did not affect I_{FRET}/I_{CFP}. Some of these compounds were fluorescent (based on the emission intensities in the CFP and YFP channels) and were discarded as interfering compounds.

A compound was considered a “hit” and kept for further analysis if it satisfied all three of the following criteria:

1. It reproducibly (n = 2) reduced I_{FRET}/I_{CFP} to a level in between the positive control average value minus two standard deviations and the negative control average value minus two standard deviations.

2. It reproducibly (n = 2) reduced the I_{FRET} value to a level in between the negative control average value minus two standard deviations and the positive control average value minus two standard deviations.

3. It reproducibly increased the I_{CFP} value to a level in between the positive control average value plus one standard deviation and the negative control average value plus one standard deviation.

Pulldowns to detect interaction between CFP-Ran and YFP-importin-β. All reactions were performed in buffer consisting of PBS + 2 mM $MgCl_2$, 5% glycerol, 0.01% NP-40, and 1.0 mM DTT. Reaction buffer was combined with the following components in this order: CFP-Ran, RCC1, BSA, GDP or GTP, importazole or 100% DMSO, and YFP-importin-β, yielding the following final concentrations: CFP-Ran: 2.5 nM, RCC1: 20 nM, BSA: 0.1 mg/ml, GDP or GTP: 200 μM, importazole: 200 μM, YFP-importin-β: 5.0 nM. Reactions were incubated for 10 min at room temperature, 20.0 μl of S-protein agarose was added, and then incubated for an additional 30 min on a rotator before pelleting the agarose at 3,000 rpm for 1 min. The supernatant was removed followed by three washes with 500 μl reaction buffer. The S-protein pellet was resuspended in 15 μl of SDS PAGE sample buffer and boiled briefly. After spinning down the S-protein pellet, 10 μl of the sample was analyzed by SDS PAGE.

HeLa cell viability assay to obtain an IC_{50}. 10,000 actively growing HeLa cells per well were transferred to opaque white 96 well tissue culture plates at a final volume of 100 µl per well in DMEM plus 4% fetal bovine serum and 1% penicillin/streptomycin. Cells were allowed to grow at 37ºC for 24 hours. Individual wells were then treated for 12 hours with one of the following conditions: 1% DMSO, 5, 10, 20, 30, 40, 50, 60, 75, 100 µM IPZ. Following this treatment, the media was replaced and cells were treated for another 12 hours under the same conditions. The plates were then allowed to equilibrate to room temperature for 30 min, after which 100 µl of room temperature CellTiter-Glo reagent from the Promega CellTiter-Glo Luminescent Cell Viability Assay kit was added to each well. Plates were shaken for 2 min, incubated at room temperature for 10 min, then read on a luminometer. The average background signal for the plate was subtracted from the value of each individual well, and the resulting values were normalized to the signal level of the DMSO containing well.

RanGAP protection assay. All steps were performed in buffer containing 50 mM HEPES pH 7.6 with 2.5 mM $MgCl_2$. Loading the Ran with GTP: 40 nM Biotin labeled RCC1 was bound to streptavidin agarose resin for 30 min rotating at 4°C. The Biotin

labeled RCC1 was a gift of D. Halpin. The beads were washed with fresh buffer to remove any free RCC1, and 16 μM nucleotide free Ran and 51 μM GTPγP^{32} were added and Ran was allowed to load for 30 min at room temperature. The beads were then removed using a spin column, and the remaining supernatant was filtered through a Sephadex G-50 column to remove free GTPγP^{32}. The loaded Ran was then diluted to 1.6 μM for further use. Performing the assay: All reactions were performed in a final volume of 200 μL and in buffer containing 50 mM HEPES pH 7.6 with 2.5 mM $MgCl_2$. Importin-β was pre-incubated with either 0.5% DMSO or 50 μM importazole in 0.5% DMSO and 100 nM RanGTPγP^{32} at room temperature. 1 μM RanGAP was added to start each reaction, and the reaction was allowed to proceed for 5 min before the reaction was stopped using 1 ml of a solution containing 7% charcoal, 10% ethanol, 0.1 M HCl, and 10 mM KH_2PO_4. The resulting solution was spun at 10,000 RPM for 2 min in a tabletop centrifuge to pellet the charcoal, and the resulting supernatant was removed and counted for three min per sample in a liquid scintillation counter.