**Figure 4 PIGN colocalizes with SAC components during SAC activation.** Colocalization (yellow) of PIGN (green) with (**a**) MAD1, (**b**) MAD2 and (**c**) MPS1 (red) in HEK293 cells. HEK293 PIGN KO cells were transfected with pMEPuro3HAPIGN plasmid for 48 hours followed by treatment with nocodazole (100 ng/µl) for 12 hours. (**d**) Colocalization of the exon 14/15 intron-retaining mutant PIGN (mut PIGN) with MAD1. The mutant plasmid was cloned by inserting a 38bp partial intron sequence into the wild-type gene in the pMEPuro3HAPIGN plasmid via restriction enzyme digestion and re-ligation. The cells were incubated for 48 hours followed by treatment with nocodazole (100 ng/μl) for 12 hours. Cells transfected with either mutant or wild-type plasmid were fixed with 4% paraformaldehyde and treated with mouse
anti-MAD, anti-MAD2 or anti-MPS1, and rabbit anti-HA, followed by treatment with fluorescently-labeled secondary antibodies. Chromosomes were stained with DAPI (blue). Laser scanning confocal microscopy was used to visualize the stained cells. Scale bars, 2-3 µm. The cells were fixed with 4% paraformaldehyde and treated with mouse anti-MAD, anti-MAD2 or anti-MPS1, and rabbit anti-HA, followed by treatment with the respective fluorescently-labeled secondary antibodies. Chromosomes were stained with DAPI (blue). Laser scanning confocal microscopy was used to visualize the stained cells, and image analyses were conducted using the Volocity 6.3 High-performance 3D imaging software (PerkinElmer). Scale bars, 2-3µm. N.D = not determined. (**e**) PIGN loss in HEK293 cells decreased cell cycle frequency in HEK293 cells. Cell cycle frequency (1/day) was significantly lower in HEK293 KO cells ectopically overexpressing the PIGN mutant (MUT) (\*p=0.0276) or empty vector (KO) control (\*p=0.0444) compared to those expressing wild-type PIGN (WT). Mean cell counts were obtained over 3 days at 12-hour intervals in three separate experiments (n=3). The cell cycle frequency (f) was calculated using the formula derived from the formula Nt = N0 2tf where Nt is the number of cells at time t, N0 is the initial number of cells and f is the frequency of cell cycles per unit time. M. Beals, L. Gross, S. Harrell. 1999. Quantifying cell division. Error bars indicate mean and standard deviation. (**f**) Mitotic index was significantly reduced in HEK293 KO cells ectopically overexpressing mutant PIGN (MUT) (\*\*p=0.0056) or empty control vector (KO) (\*\*\*p=0.0004) compared to wildtype (WT) PIGN. Error bars are representative of the mean and standard error from the mean in three independent experiments (n=3).