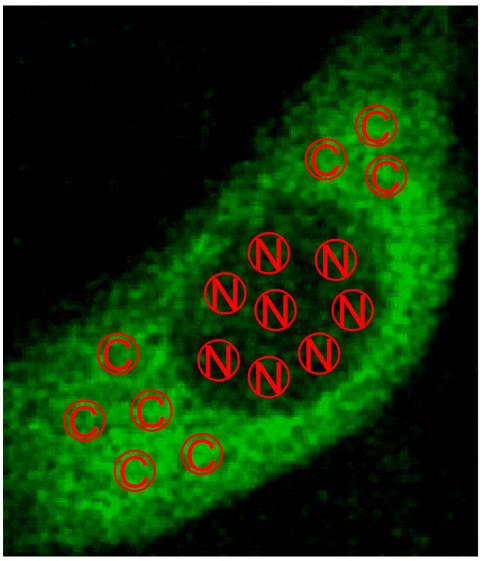
**Figure S1**. Schematic illustration of how nuclear localization index was determined. Circles with a diameter of 12-pixels radius were randomly selected as shown. The nuclear index is calculated based on the formulated provided.

**Figure S2.** Alignment of Mps1 orthologs. The sequences of human, mouse, Xenopus, and Zebrafish Mps1 were aligned.

**Figure S3.** The localization of siRNA resistant Mps1 and Mps1 mutants at the $G_2$/M boundary upon depletion of the endogenous Mps1. The cells expressing siRNA-resistant Mps1 WTR, M8R, M9R, and N60N61R were transfected with the Mps1 siRNA twice prior to synchronization by R0–3306 at the $G_2$/M boundary. The subcellular localization of Mps1 and the Mps1 mutants were detected by immunofluorescence.



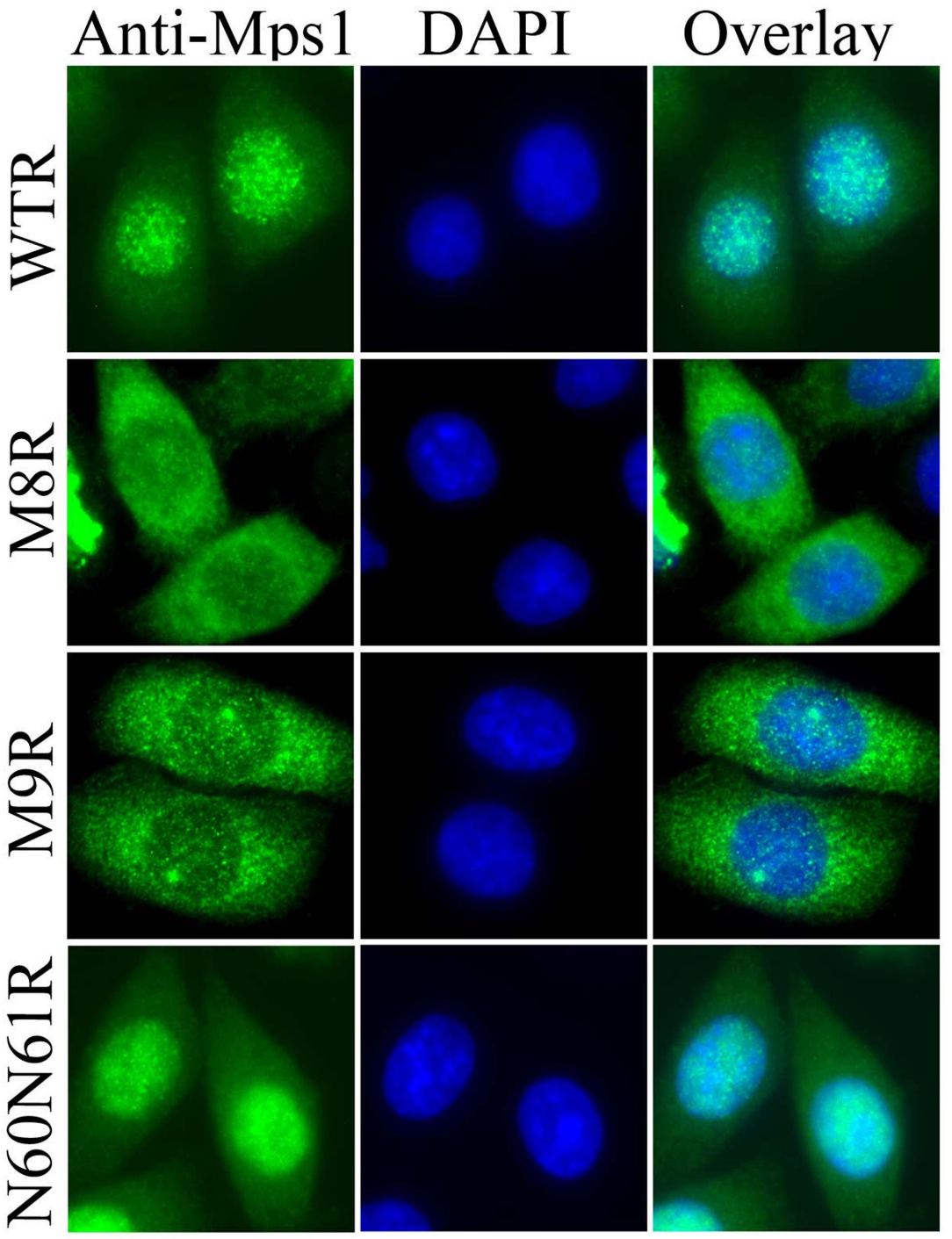

Ten circles were selected randomly in nuclear or cytoplasm via ImageJ and the responding fluorescence of each circle was counted. The nuclear localization index was calculated as:

Nuclear localization index = $\overline{Fn}/(\overline{Fc} + \overline{Fn})$

$\overline{Fn}$: average fluorescence of nuclear circles

$\overline{Fc}$: average fluorescence of cytoplasmic circles


LXXLL Motif
Inverted LXXLL Motif
Human 65-W L S L L L K L E K N S V P L S D A-- L L N K L I G-89
Mouse 61-W L N F L L K L E K N S S P L N D D-- L L N K L I G-85
Xenopus 65-W L N C L L K L E N T G L P Q I D P Q L L N K L I D-90
Zebra fish 47-C R T F L S N L E K K G N P Q A D P S L L S K L M D-72


Anti-Mps1
DAPI
Overlay
WTR
M8R
M9R
N60N61R