Figure Legends:

SUPPLEMENTARY FIGURE 1. GFP-M6(K157R), like wild-type myo6, targets to uncoated vesicles. **(A)** Localization of the transferrin receptor (TsfnR) in GFP-M6 and GFP-M6(K157R) transfected cells. The boxed areas in the left hand panels are enlarged in the right hand panels. Arrows point out overlap between the GFP constructs and TsfnR-containing vesicles found in cell peripheries. **(B)** Indirect immunofluorescence staining of clathrin heavy chain in GFP-M6(K157R) transfected cells reveals no significant overlap. The boxed area is enlarged in the right hand panels. Scale bars represent 10 μm in left hand panels, and 2.5 μm in right hand panels.

SUPPLEMENTARY FIGURE 2. Overexpression of the myo6 motor domain mutant, GFP-M6(K157R), blocks trafficking of transferrin to the early endosome. GFP-M6, GFP-M6tail, and GFP-M6(K157R) transfected cultures were incubated with Rhodamine-conjugated transferrin (R-Tsfn) for either 15min or 30min. Transfected cells lacking perinuclear staining are demarcated by closed arrows whereas transfected cells that accumulate transferrin normally are shown with open arrows. Scale bars represent 10μm.

SUPPLEMENTARY FIGURE 3. Overexpression GFP-M6(K157R) blocks trafficking of transferrin at the uncoated vesicle stage. ARPE-19 cells transfected with GFP-M6, GFP-M6tail, or GFP-M6(K157R) were surface labeled with R-Tsfn at 4^{o}C (0 min) and then chased for 2min,10min, or 30 min at 37^{o}C. **(A)** Quantitation of the percentage overlap between GFP positive structures and endocytosed R-Tsfn during the course of the pulse-chase experiment. The standard deviation represents the average of three experiments. **(B)** Representative fields showing the overlap between GFP-M6(K157R) (green) and R-Tsfn (red) after 0min, 2min or 10 minutes of chase. Each image is oriented with the nucleus to the left, and the cell edge to the right. Overlap between the two components is seen as yellow and is found maximally after >2min uptake. Scale bar = 10 μm.

Supplementary Figure 3:

GFP-M6(K157R) blocks trafficking at the uncoated vesicle stage.

We confirmed that the expression of GFP-M6(K157R) was blocking trafficking at the uncoated vesicle stage, and not a different step in the endocytic pathway, in a series of pulse-chase and immunolocalization experiments. Expression of GFP-M6(K157R) had no apparent effect on the number or distribution of clathrin coated pits, as judged by immunolocalization of Clathrin (Supplementary Figure 1B) and the clathrin adapter AP-2 (not shown) in transfected cells. Furthermore, when the clathrin-coated pits of GFP-M6(K157R)-transfected cells were surface labeled by incubation with R-Tsfn at 4^{o}C, there was no apparent difference in transferrin receptor density nor clathrin-coated pit density when compared to GFP-transfected cells (data not shown). Therefore, GFP-M6(K157R) expression did not affect clathrin-coat assembly or

transferrin-receptor clustering into pits.

To determine the stage at which endocytosis was blocked in GFP-M6(K157R)-transfected cells, we surface labeled cells with R-Tsfn at 4^{o}C then warmed them to 37^{o}C for 2-30 minutes and compared the location of the endocytosed transferrin to the location of the GFP-tagged proteins. At the zero timepoint, only 6.6 ± 0.5% of GFP-M6(K157R)-associated vesicles colocated with R-Tsfn (Supplementary Figure 3A,B). This level of overlap is similar to what was observed for both wild-type GFP-M6 and GFP-M6tail (our unpublished data; Aschenbrenner et al., 2003), confirming that none of the myo6 constructs targeted to clathrin-coated pits.

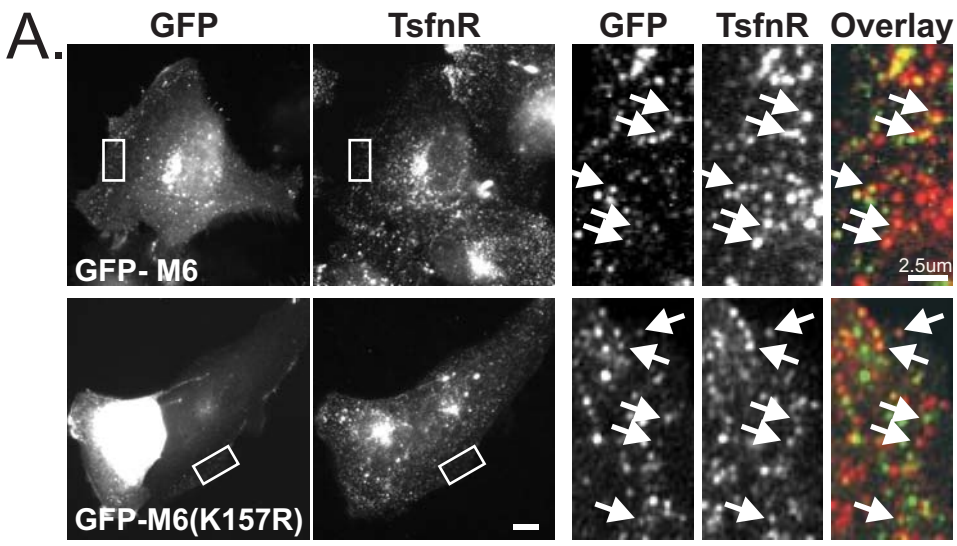
Maximal overlap between the endocytosed transferrin and GFP-(M6K157R) was observed following 2 mins chase at 37^{o}C (Supplementary Figure 3A,B). 41.7 ± 1.3% of the GFP-M6(K157R)-labeled vesicles contained endocytosed R-Tsfn suggesting that the myo6 motor mutant, like full-length myo6 and the tail domain of myo6 (Aschenbrenner et al., 2003), is recruited to endocytic vesicles immediately after uncoating. This high level of overlap was maintained after 10 minutes of further chase at 37^{o}C (45.0 ± 6.1%; Supplementary Figure 3A,B), suggesting a block at the uncoated vesicle stage similar to that seen for GFP-M6tail expressing cells (Aschenbrenner et al., 2003).

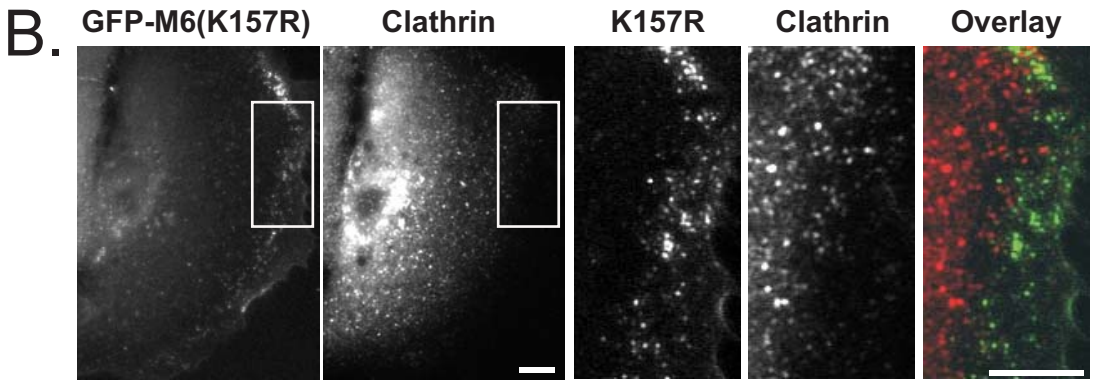
Both vector analysis and steady-state uptake studies showed that GFP-M6tail-associated vesicles are able to exit from the peripheral actin network and achieve early endosome fusion given sufficient time. In agreement, after 30 minutes of chase at 37^{o}C the overlap between transferrin and GFP-M6tail returned to baseline levels (Supplementary Figure 3A; Aschenbrenner et al., 2003). In contrast, GFP-M6(K157R) associated vesicles continued to exhibit a high degree of overlap with the endocytosed R-Tsfn (47.2 ± 2.3%; Supplementary

Figure 3A,B). These vesicles were not early endosomes as judged by the lack of overlap with the early endosome marker, EEA1 (Figure 1C). Therefore, binding to the actin cytoskeleton effectively blocks trafficking of uncoated vesicles to the early endosome.

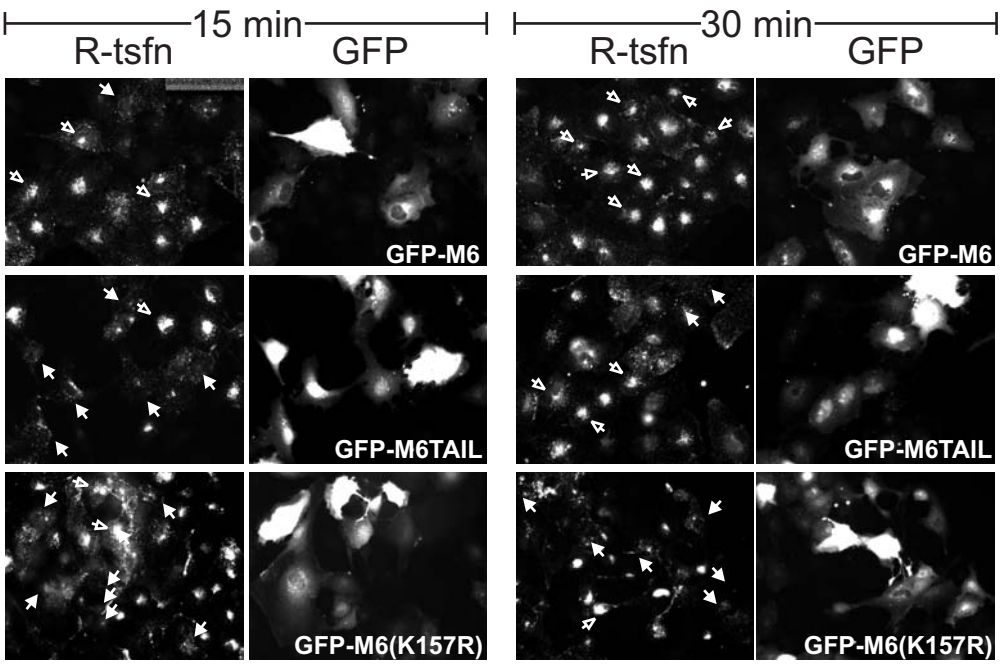
REFERENCES:

Aschenbrenner, L., T. Lee, and T. Hasson. 2003. Myo6 facilitates the translocation of endocytic vesicles from cell peripheries. *Mol. Biol. Cell*. 14:2728-2743.





Supplemental Figure 1
Aschenbrenner, Naccache & Hasson



Supplemental Figure 2.
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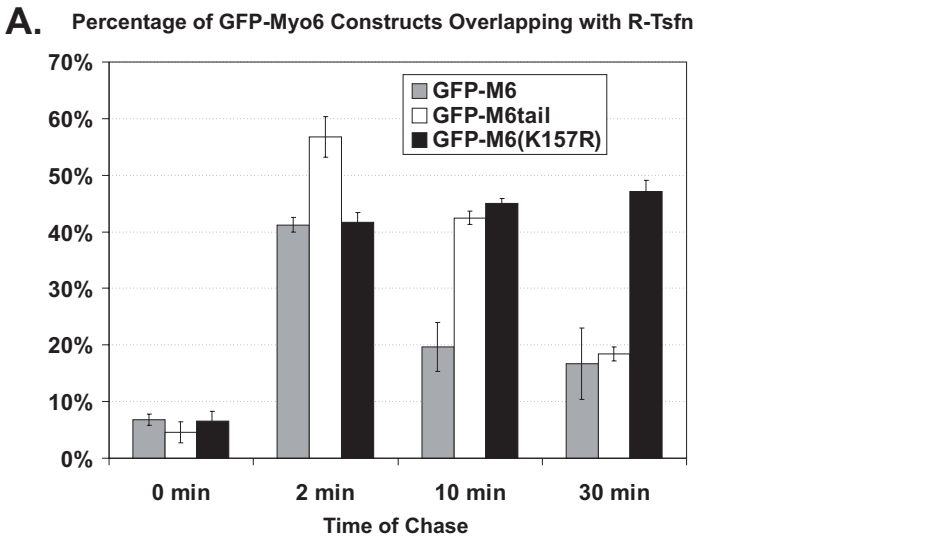

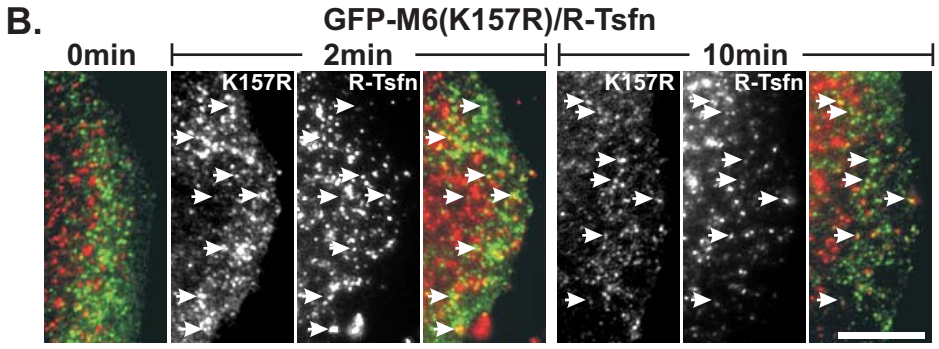

Supplemental Figure 3
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