

Characterization of a Novel Mutation in the COPI Vesicle on Binding to Dilysine Motifs

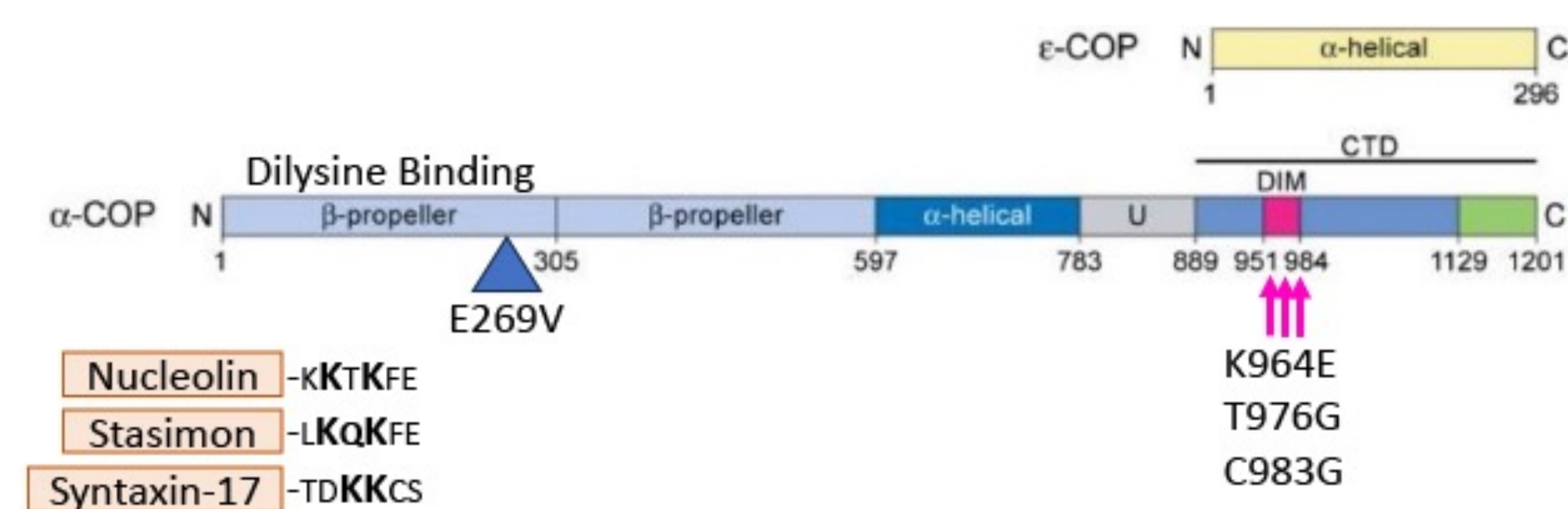
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BACKGROUND

The heptameric COPI coatomer complex is involved in the formation of vesicles and the intracellular trafficking of proteins between the Golgi and Endoplasmic Reticulum and throughout the cytoplasm. Members of the COPI complex bind dilysine motifs found in the C-terminal domain of cargo proteins, particularly KKxx or KxKxx. We generated a point mutation in the WD40 domain COPI alpha subunit (α -COP), which we predicted would impair its ability to bind KxKxx cargo. When expressed in HEK-293TT cells, this mutant no longer co-immunoprecipitated (co-IP) the KxKxx-containing RNA binding protein Nucleolin (NCL) or the microtubule-associated protein Stasimon.

HYPOTHESIS

We hypothesized that the mutation of the α -COP N-terminal WD-40 domain would be unable to bind cargo proteins terminating with the dilysine domain KxKxx in Nucleolin and Stasimon/Tmem41b but would bind the KKxx in FLAG-Syntaxin17. We predicted that a mutation in the α -COP C-terminus that impairs interaction with ϵ -COP would not affect the ability to bind dilysine-containing cargo.



METHODOLOGY

HEK-293TT cells were transfected with wild-type and mutants of α -COP. One mutant is in the N-terminal WD-40 domain at amino acid 269 changed from glutamic acid to valine (E269V). The second mutant is in the C-terminal domain (3X) and eliminates binding to the ϵ -COP COPI subunit. Wild-type and mutant α -COP were immunoprecipitated using anti-MYC beads. Endogenous Nucleolin was immunoprecipitated using Protein A beads conjugated to rabbit anti-Nucleolin antibody. Western blots of inputs and immunoprecipitates of each experiment were conducted to determine the ability of α -COP to bind dilysine-containing proteins.

RESULTS

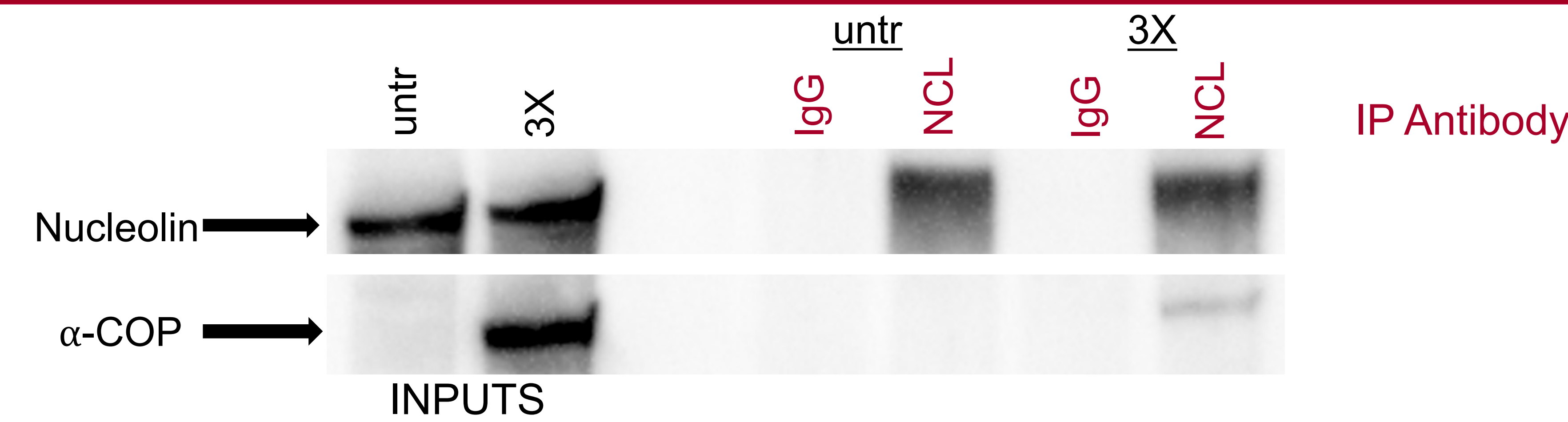


Figure 1. Antibody Validation and Preliminary Nucleolin/ α -COP co-IP

We first validated the specificity of the Nucleolin antibody to IP endogenous Nucleolin. The IPs in the top panel show that no Nucleolin IP'd with control rabbit IgG alone while a strong signal was present when NCL antibody was used. To determine if the 3X α -COP mutant co-IP'd Nucleolin, HEK-293TT cells were either untransfected (untr) or transfected with myc/FLAG-tagged 3X α -COP. The inputs show that 3X α -COP was expressed and expression did not alter the levels of endogenous Nucleolin. The bottom panel shows that 3X α -COP DID co-IP with endogenous Nucleolin.

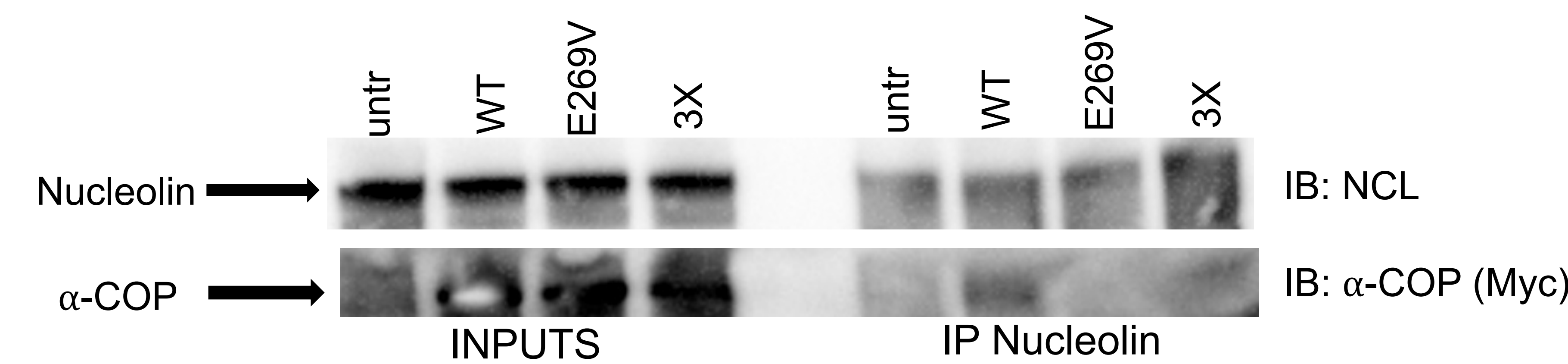


Figure 2. Endogenous Nucleolin/ α -COP co-IP

To confirm the 3X α -COP mutant co-IP'd Nucleolin, HEK-293TT cells were transfected with WT, E269V, or 3X α -COP. The IPs in the top right panel show that endogenous Nucleolin was successfully pulled down. The co-IPs in the bottom panel show that the transfected WT α -COP and 3X α -COP co-IP'd with Nucleolin. As expected, the E269V α -COP mutant did not co-IP with Nucleolin.

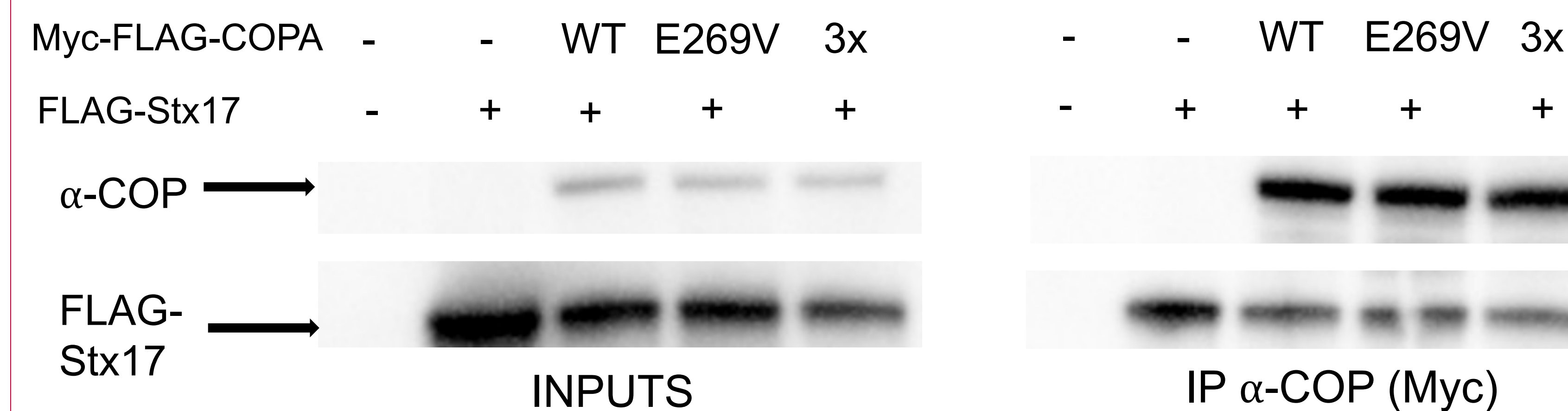


Figure 3. FLAG-Syntaxin17 non-specifically binds to Myc magnetic beads

To determine if either E269V or 3X mutant α -COP affected binding to KKxx cargo, HEK-293TT cells were either untransfected or transfected with WT, E269V, or 3X and FLAG-Syntaxin17 α -COP. As seen in the second IP lane of the bottom right panel, FLAG-Syntaxin17 non-specifically binds to the Myc beads. To troubleshoot the non-specific binding of FLAG-Syntaxin17 to the Myc magnetic beads, the 0.5% NP40 washes were increased to 1%, but there were no improvements in the background. For future experiments, additional high salt washes with 0.5M NaCl will be added to the protocol.

RESULTS

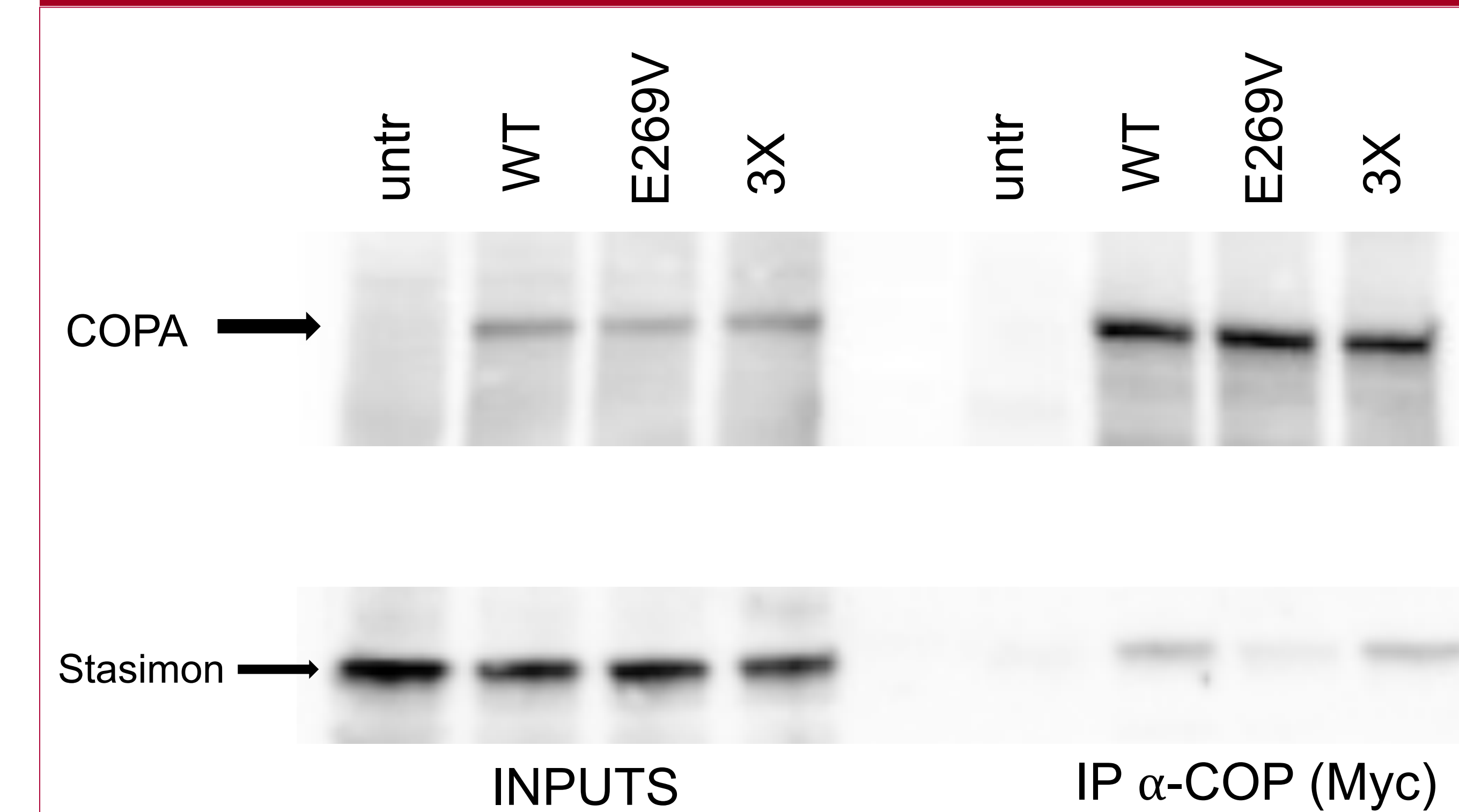


Figure 4. Endogenous Stasimon(Tmem41b)/ α -COP co-IP

To determine if the E269V or 3X α -COP mutants affect binding to the KxKxx-containing protein Stasimon/Tmem41b, HEK-293TT cells were either untransfected (untr) or transfected with WT, E269V, or 3X α -COP. The IPs in the top right panel show successful expression and IP of Myc-tagged WT, E269V, or 3X α -COP. The IPs in the bottom right panel show that endogenous Stasimon DID co-IP with WT and 3x α -COP. As hypothesized, very little Stasimon co-IP'd with E269V α -COP. These data show that the E269V mutation impairs the ability of α -COP to bind these KxKxx-terminating COPI cargos.

Conclusion

The inability of mutation E269V to co-IP dilysine proteins implies that the WD40 domain of the COPI α -COP protein is required for binding to KxKxx-terminating proteins, as typified in Nucleolin and Stasimon.

The C-terminal 3X mutation shows that ϵ -COP is not necessary for dilysine recognition and implies that α -COP directly binds to this KxKxx motif.

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