Supplementary Material

Wu and Wolley *et al.*, Effect of oral potassium supplementation on the thiazide-sensitive sodium chloride cotransporter in humans: a randomized crossover trial

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Supplementary Methods

- **Sample size**

The sample size calculated for this study was based on a t-test designed to examine the association between oral KCl supplementation and uEV levels of NCC and pNCC in healthy adults. In a human study of 4 subjects with a very low (potassium <50mmol/day) vs. high (>120mmol/day) potassium diet (4 days each phase), pNCC was 2-fold higher in the low potassium phase [1]. Therefore, for comparisons we would assume a minimum of ≥1.5-fold difference between groups (with a variance of <0.2 from our experience) and we calculate that ≥10 patients in each group would be required to detect this magnitude of difference at a power of 80%.

- **Eligibility**

Inclusion criteria: Eligible participants were willing and able to adhere to consuming the meals, snacks, and supplements (Span K or placebo) provided across the dietary intervention periods of the study; living in an area where pre-prepared meals could be delivered; and able to store the pre-prepared meals in a refrigerator. Any other dietary supplements usually taken by the participant needed to be ceased for the duration of the study.

Exclusion criteria: Participants were ineligible if they had conditions including hypertension or use of anti-hypertensive drugs or diuretics, heart failure or chronic kidney disease or a relatively high plasma K^+ concentration (≥5.0 mmol/L) at enrolment; a food allergy or intolerance which could not be accommodated for in the study foods provided or coeliac disease; were vegan or vegetarian or currently following a restrictive or weight loss diet; or unable to abstain from drinking alcohol during the study diet periods.

- **Analyses inclusion and exclusion criteria**

Participants were included if their 24 h urinary Na^+ excretion rates were reasonably concordant (~180 mmol/d, as urinary sodium typically reflecting 90% of dietary intake[2]) with the prescribed daily dietary sodium intake (200 mmol/d), urinary K^+ excretion rose as expected between the placebo and active KCl supplement phase, and their uEVs were successfully isolated and NCC and pT60-NCC were detected from both phases. Participants were excluded if their urinary Na^+ did not reasonably match the prescribe amount (as defined above), urinary K^+ in the active phase did not rise in response to supplemental KCl administration, their uEV were not successfully isolated, or NCC and pT60-NCC were not detected from both phases.

- **Description of dietary intervention**

Prior to each phase commencing, eligible participants were provided with electronic and hard copies of their meal plan as a guide for which meals, snacks, and drinks to consume on each day. The participants were instructed to use the meal plan to record compliance with the food and drinks as well as the study supplements prospectively, by circling the amount of each meal, snack and drink they consumed and listing any additional food or drinks (outside of water) consumed. Adherence to each meal plan was calculated as a percentage based on the proportions of each meal and snack consumed compared to the total amount prescribed. Adherence was also calculated separately for the final 24 h of each diet period (i.e., the period which crossed over with the urine and blood pressure data collection).

All foods were provided to participants throughout the trial, and meal plans were created individually for each participant by an Accredited Practicing Dietitian. Participants' daily energy requirements were estimated to achieve energy balance for weight maintenance based on the participant's self-reported usual dietary intake and physical activity levels, height, weight and sex. After the run-in phase, participants provided feedback on the amount and types of meals and snacks included and adjustments were made to the meal plans for the intervention phases if needed. The meal plans were high in Na^+ and lower than currently recommended [3](hereafter designated "low") in K^+ including an estimated average of 4,500 mg/day Na^+ (~200 mmol) and 2,300mg /day K^+ (~60 mmol), with some variation across participants relative to their energy content and preferences. All meal plans consisted of breakfast, lunch and dinner pre-prepared fresh meals and some packaged snacks purchased from Youfoodz Pty Ltd (Brisbane, Australia) which were delivered to the participant's home. Most meal plans also included additional packaged snacks purchased from local supermarkets provided to the participants by the study coordinator at appointments. The estimated energy, Na^+ and K^+ content of Youfoodz meals and snacks were provided by the company based on analyses in Nutritics software (Dublin, Ireland) that utilised the Australian food composition database AUSNUT 2016. For additional snacks the estimated energy and Na^+ content was determined by the food package labelling and the K^+ content using Foodworks 10 software (Xyris Pty Ltd, Brisbane, Australia). If the participant usually consumed coffee, tea or an alternative such as hot chocolate daily, these were included within the meal plans and counted in the estimated nutritional content allowances (including specific milk content where applicable), but were provided by the participant. Including these beverages was allowed to ensure participant compliance. Participants were otherwise advised to only consume water and no additional foods. For the 2-day washout, participants were able to consume their usual diet to enhance likelihood of compliance across the study intervention periods.

During the two intervention phases, participants consumed 3 tablet supplements each at breakfast, lunch and dinner meals, with a total of 9 supplements daily for Sunday (Day 1) to Thursday (Day 5), and 3 supplements on Friday (Day 6) morning. The high K^+ intervention consisted of all food provision with addition of KCl

supplementation (3×Span K [600 mg KCl] tablet 3 times daily, total additional 72 mmol K^+ per day), increasing the average estimated K^+ intake to ~132 mmol per day, while the low K^+ intervention was the same diet and placebo tablets (3×placebo 3 times daily).

- **Sample measurements**

Measurements of blood and 24 h urine were performed by Pathology Queensland Laboratory immediately after collection was completed. Plasma aldosterone was determined by LC-MS/MS[4] and direct renin concentration was measured by chemiluminescent immunoassay[5]. Spot urine creatinine concentration was measured in the EHRC research laboratory, using a creatinine urinary detection assay kit (EIACUN, Invitrogen, US).

- **Nanoparticle Tracking Analysis (NTA)**

NTA, a NanoSight NS500 instrument (Nanosight Ltd, UK) with NanoSight NTA v3 software was used. Before each session, the acquisition parameter settings were determined using the NTA Latex Microsphere 100nm (Malvern Polystyrene, UK) in a 1/250 dilution in Ultrapure water (Pureau, AU) and fixed for all measurements during the session [camera level 10, slider shutter 696, slider gain 73, detection threshold 5]. The uEV pool was analysed on 5 captures of 60 seconds. Sample dilution was 1/1000 to obtain the recommended number of particles (50-100) per image.

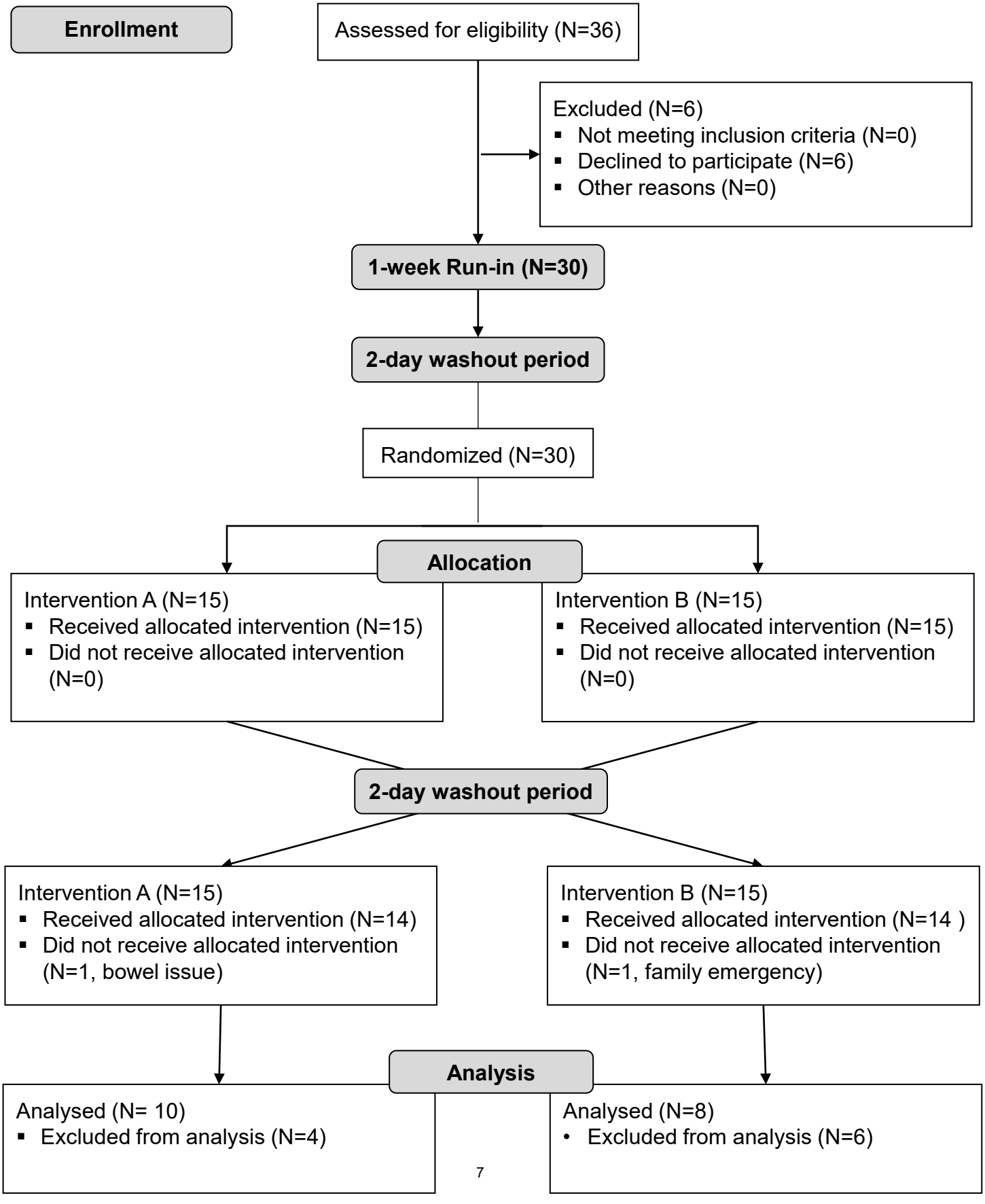
Table S1. List of antibodies in use.

Antibodies	Manufacturer	Dilution	Measurement
Rabbit anti-NCC	AB3553, Merck Millipore, Germany	1/1000	Inferred by measurement of the dominant band between 100-150kDa.
Rabbit anti-T60pNCC	Robert A Fenton's Lab, Aarhus, Denmark [6]	1/2500	Inferred by measurement of the dominant band between 100-150kDa.
Rabbit anti-phospho-SPAK	07-2273, Sigma-Aldrich, US	1/500	Measured by the dominant band of ~60kDa.
Rabbit anti-ALIX	ABC40, Merck Millipore, Germany	1/2000	Measured by the dominant bands of 96kDa.
Rabbit anti-TSG101	MASBC649, Merck Millipore, Germany	1/2000	Measured by the dominant bands of 45kDa
Rabbit anti-CD9	ab92726, Abcam, UK	1/1000	Measured by the dominant bands of 45kDa
HRP conjugated goat anti-rabbit IgG	12-348, Merck Millipore, Germany	1/20,000	/

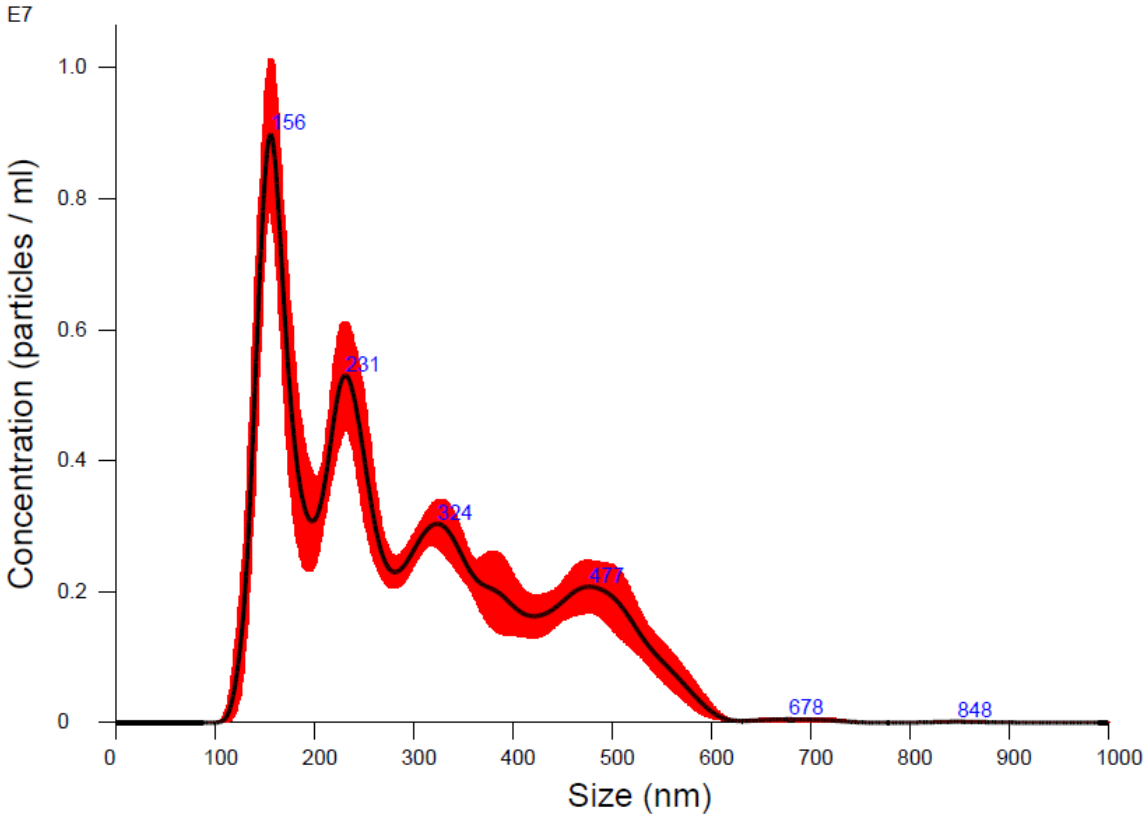
Supplementary Table S2. Participants included in analyses. Y, included in analysis; N, excluded in analysis; NA, not applicable.

Subject#	Biochemistry and BP analyses		uEV NCC analyses				Met criteria and included in analyses	uEV pSPAK included in analyses
	Dietary compliance >80%	Urinary Na+ and K+ response	Provided adequate urine volume for uEV isolation	Successful isolation of uEV in both intervention phases	Detection of NCC in both intervention phases	Detection of pNCC in both intervention phases		
1	Y	Y	Y	Y	Y	Y	Y	Y
2	Y	Y	Y	Y	Y	Y	Y	Y
3	Y	Y	Y	Y	Y	Y	Y	N
4	Y	Y	Y	Y	Y	Y	Y	N
5	Y	Y	Y	Y	Y	Y	Y	Y
6	Y	Y	Y	Y	Y	Y	Y	Y
7	Y	Y	Y	Y	Y	Y	Y	Y
8	Y	Y	Y	Y	Y	Y	Y	N
9	Y	Y	Y	Y	Y	Y	Y	N
10	Y	Y	Y	Y	Y	Y	Y	Y
11	Y	N	Y	Y	Y	Y	N	N
12	Y	Y	Y	Y	Y	Y	Y	N
13	Y	Y	Y	Y	Y	Y	Y	N
14	Y	Y	Y	N	N	N	N	N
15	Y	Y	Y	Y	Y	Y	Y	N
16	N	N	Y	Y	Y	Y	N	N
17	Y	Y	Y	Y	Y	Y	Y	Y
18	Y	Y	Y	Y	N	N	N	N
19	Y	Y	N	NA	NA	NA	NA	NA
20	Y	Y	N	NA	NA	NA	NA	NA
21	Y	N	Y	Y	Y	Y	N	N
22	Y	Y	N	NA	NA	NA	NA	NA
23	N	N	Y	Y	Y	Y	N	N
24	Y	Y	Y	Y	Y	Y	Y	N
25	Y	Y	Y	Y	Y	Y	Y	Y
26	Y	Y	Y	Y	Y	Y	Y	Y
27	N	N	Y	N	Y	Y	N	N
28	Y	Y	Y	Y	Y	Y	Y	N
Sum							Y18/N7/NA3	Y9/N16/NA3

Supplementary Figure S1. The CONSORT flowchart of the double-blind, randomized and placebo-controlled cross-over trial. Intervention A, 5-day of high sodium (Na^+) low potassium (K^+) diet supplemented with Span K; Intervention B, 5-day of high Na^+ low K^+ diet supplemented with placebo.



Supplementary Figure S2. Nanoparticle tracking analysis of uEV pool.



Supplemental Figure S3 (A-L) . Immunoblots of analysed proteins in all participants.

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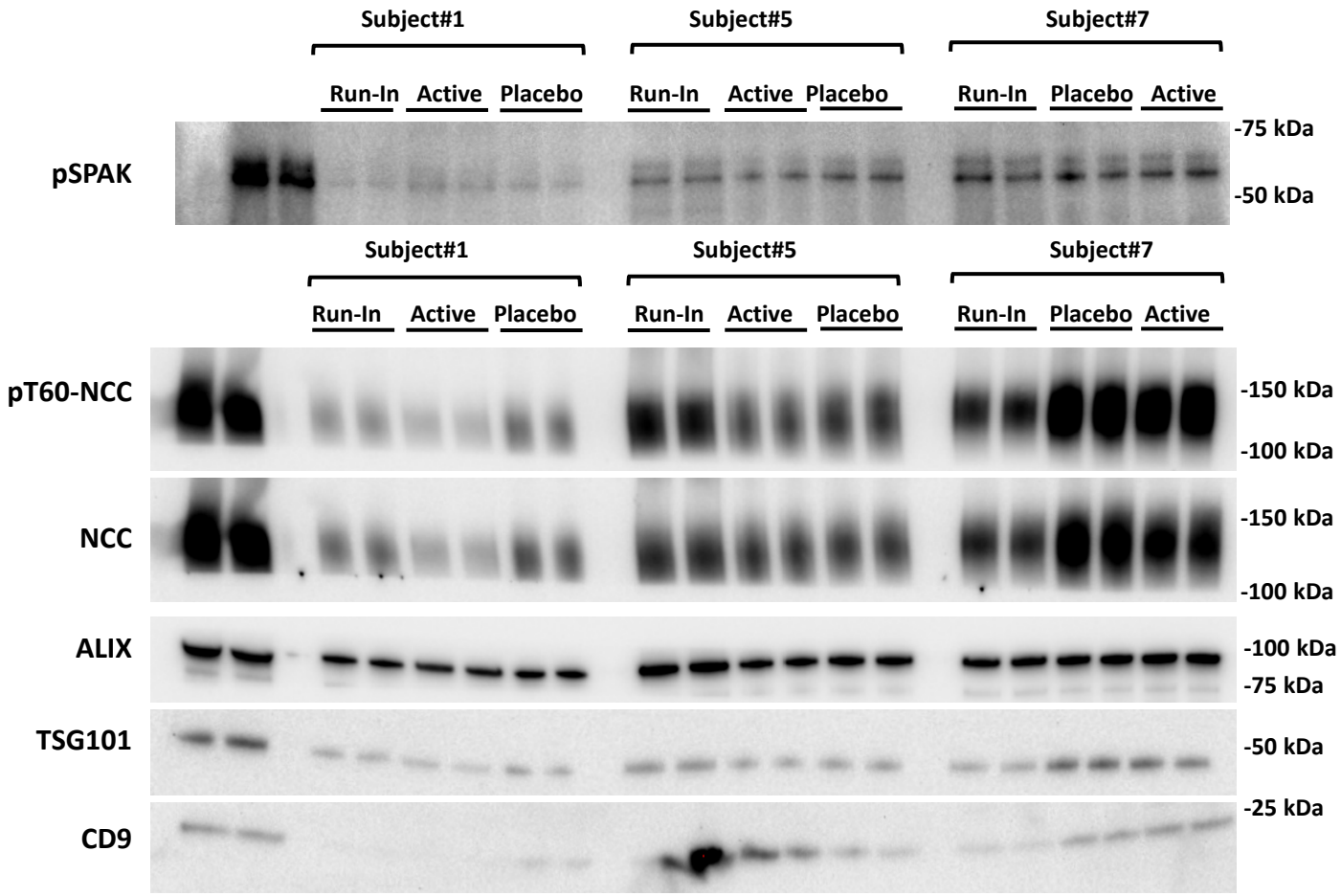

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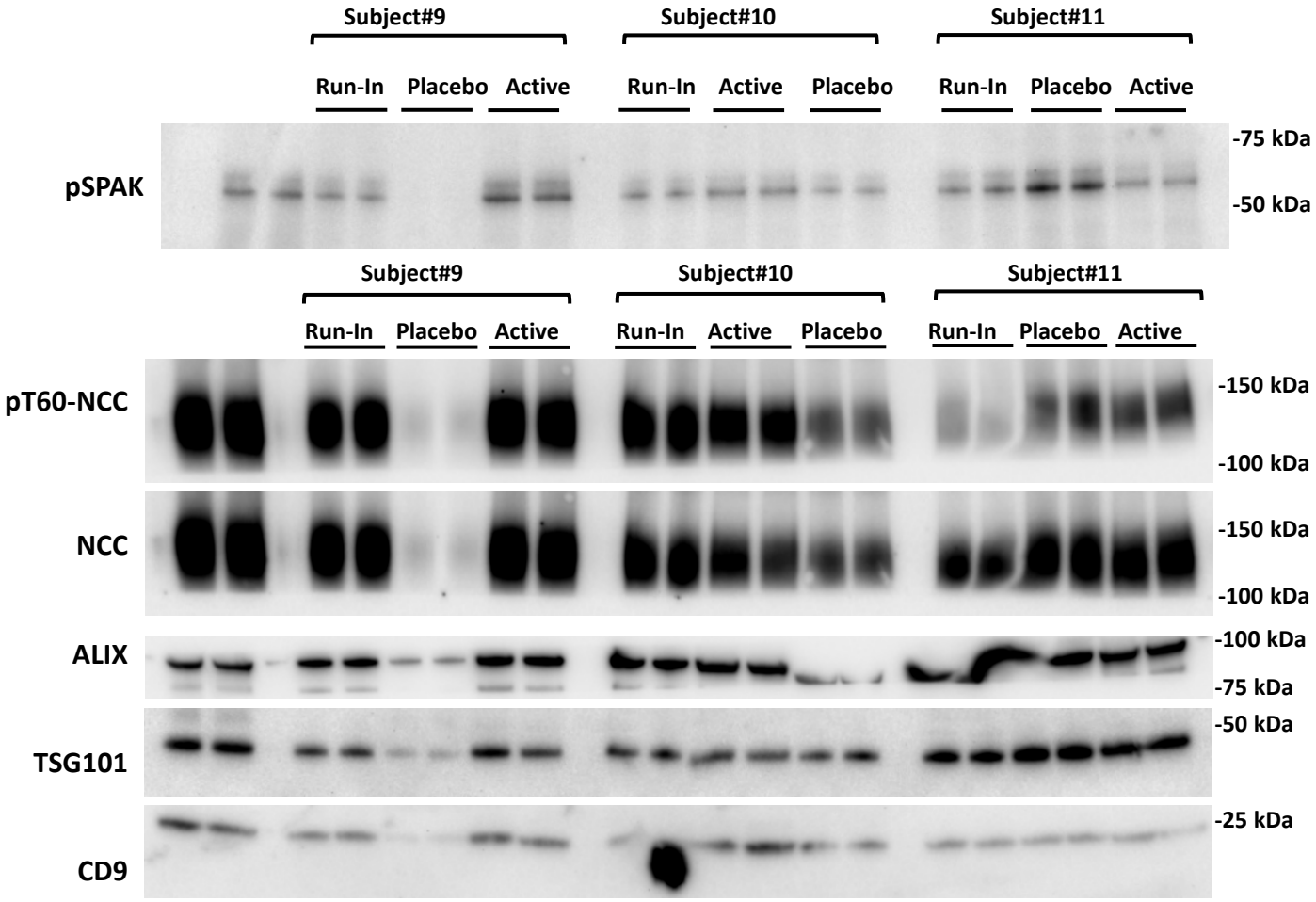
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Active
Run-In
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Placebo
Run-In
Placebo
Active
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-50 kDa
Subject#9
Subject#10
Subject#11
Run-In
Placebo
Active
Run-In
Active
Placebo
Run-In
Placebo
Active
pT60-NCC
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-100 kDa
NCC
-150 kDa
-100 kDa
ALIX
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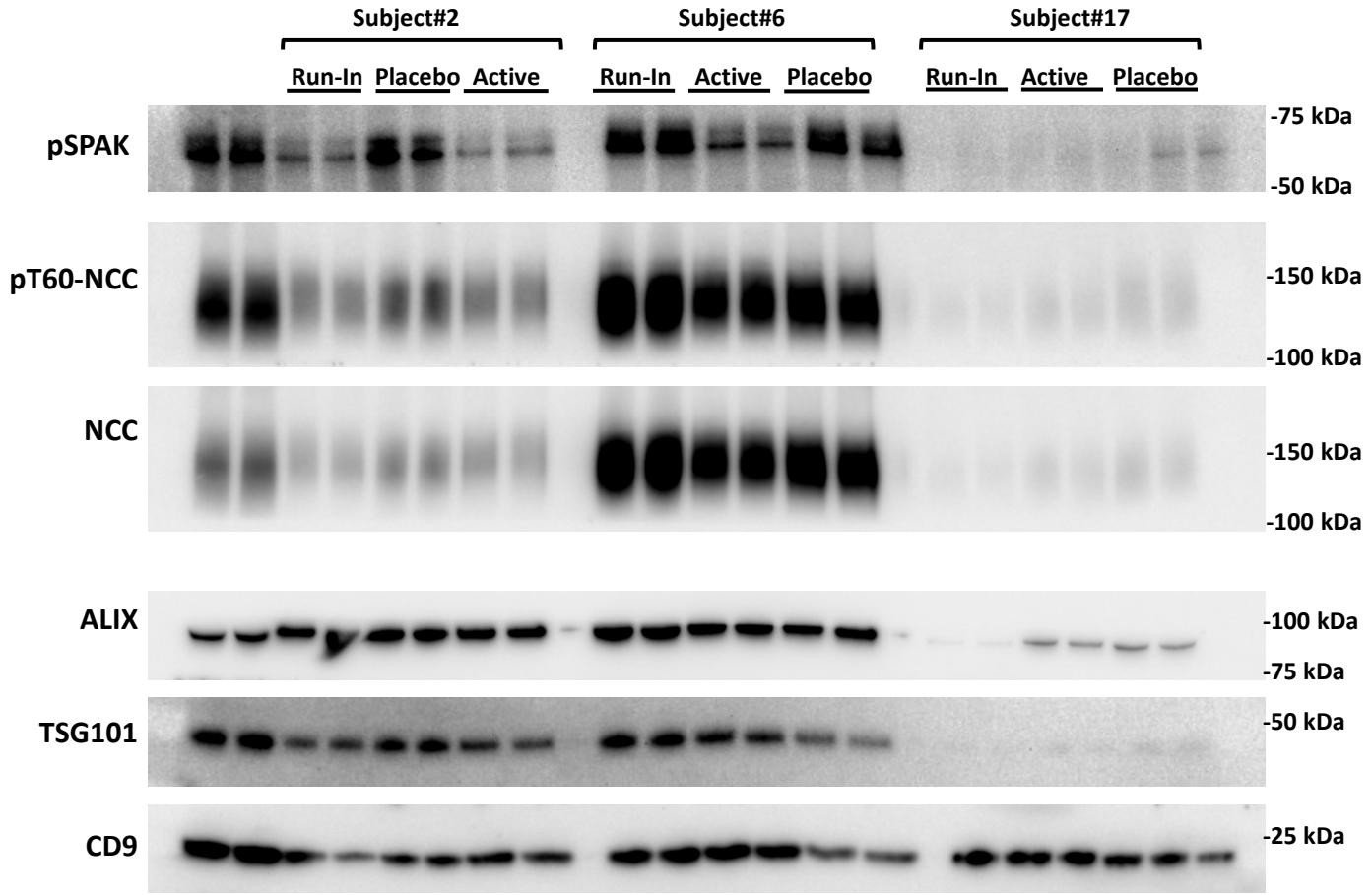
TSG101
-50 kDa
CD9
-25 kDa

C.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pT60-NCC
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ALIX
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TSG101
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CD9
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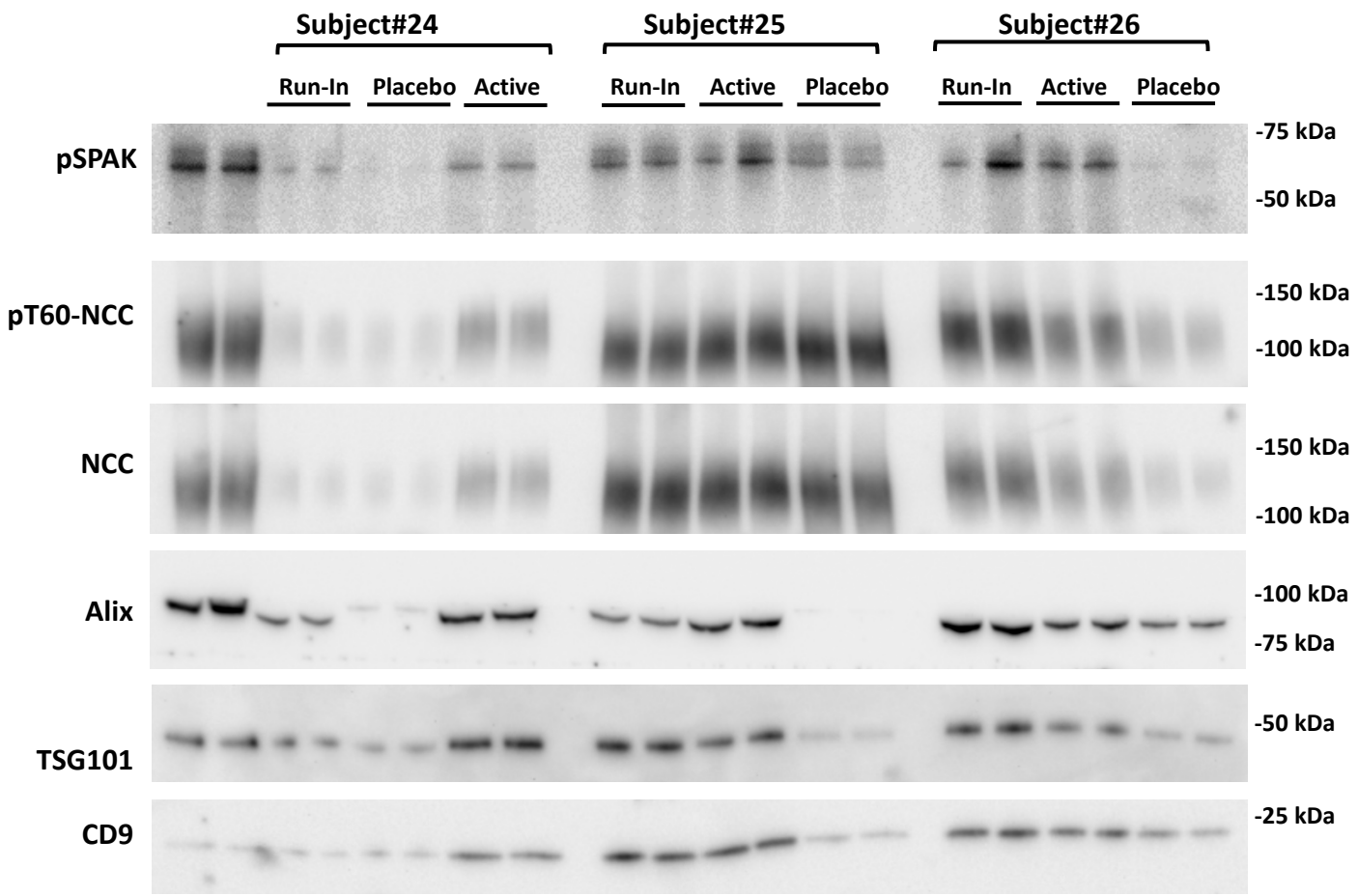
D.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pT60-NCC
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CD9
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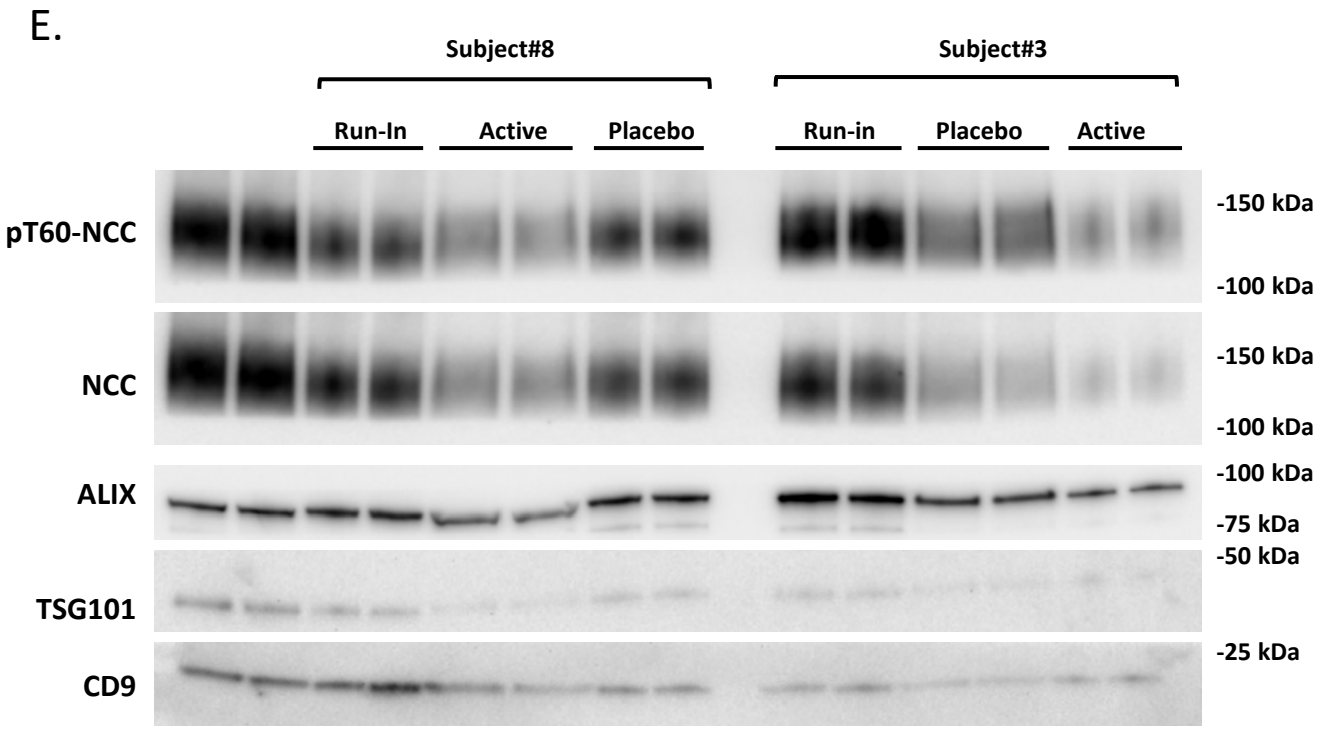
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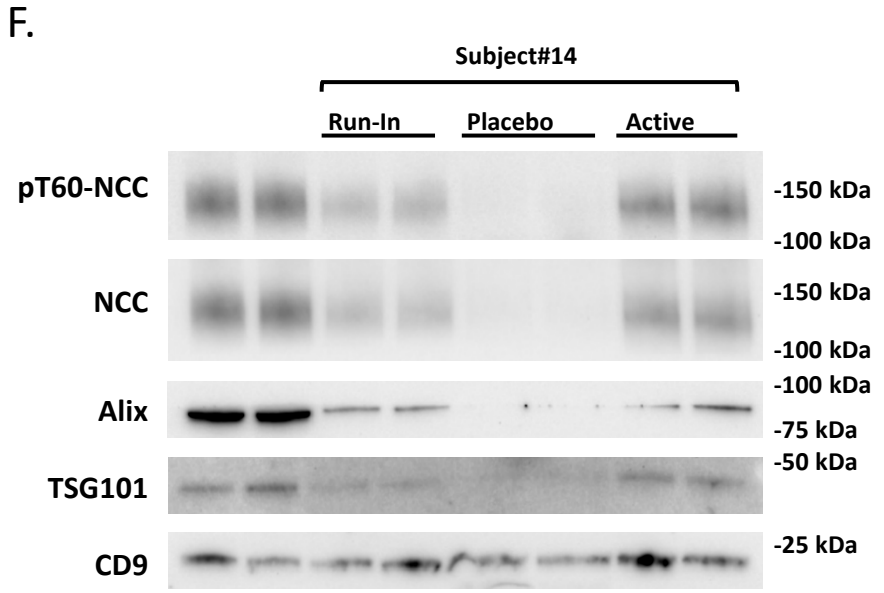
-50 kDa
-25 kDa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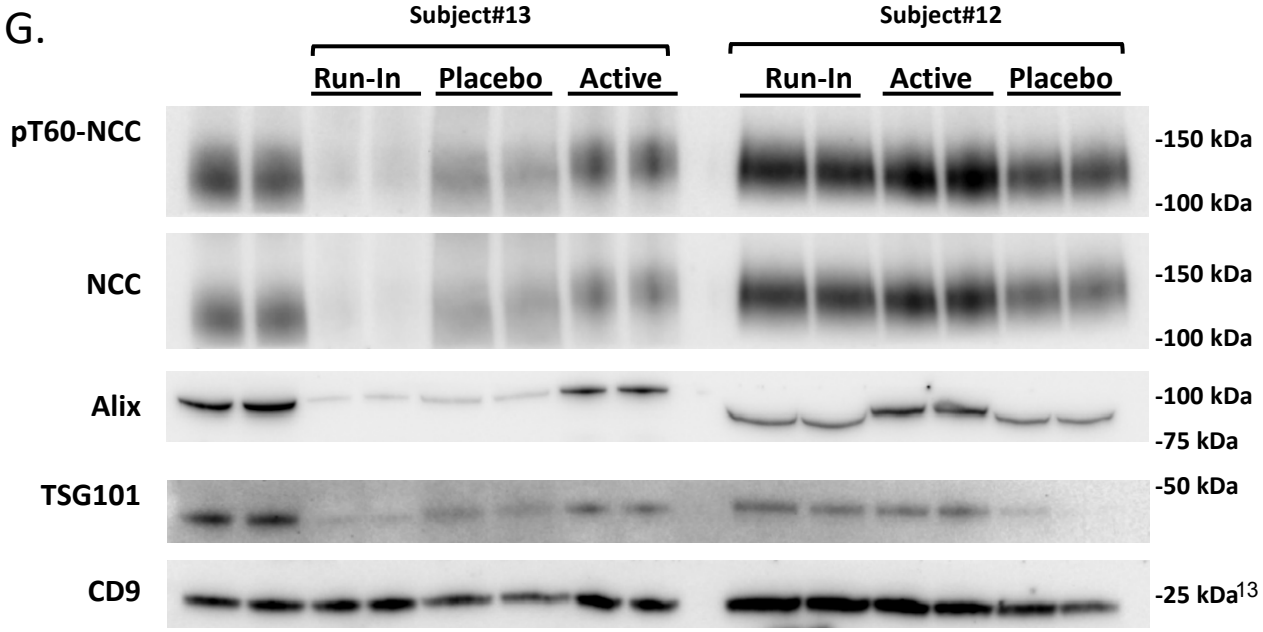
-25 kDa


G.
Subject#13
Subject#12
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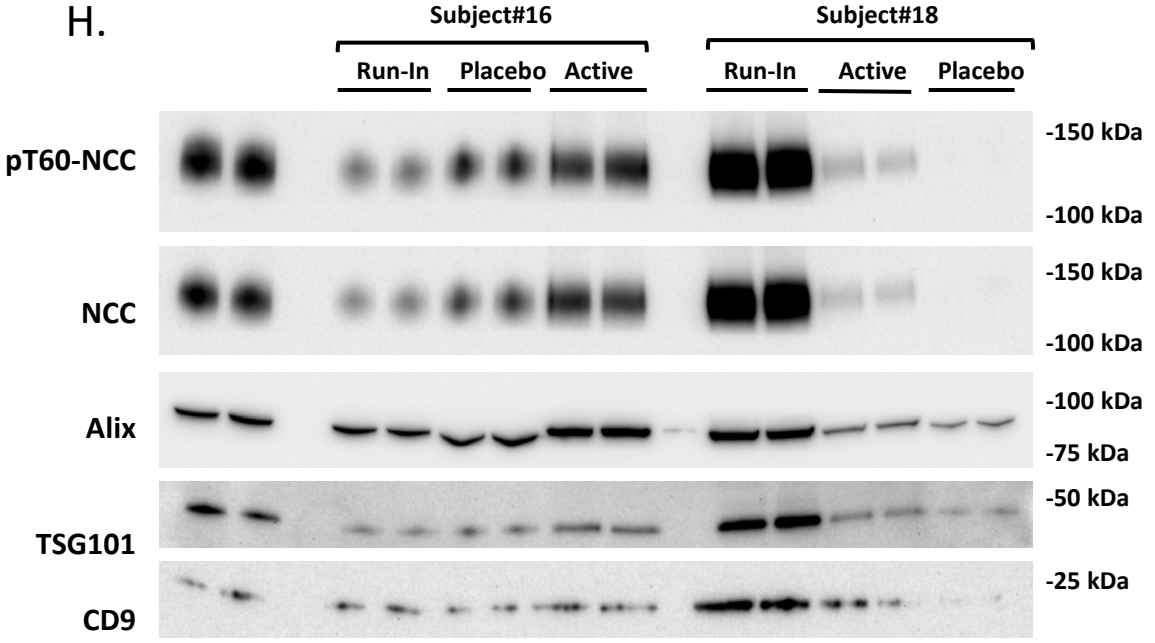
-25 kDa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Subject#18
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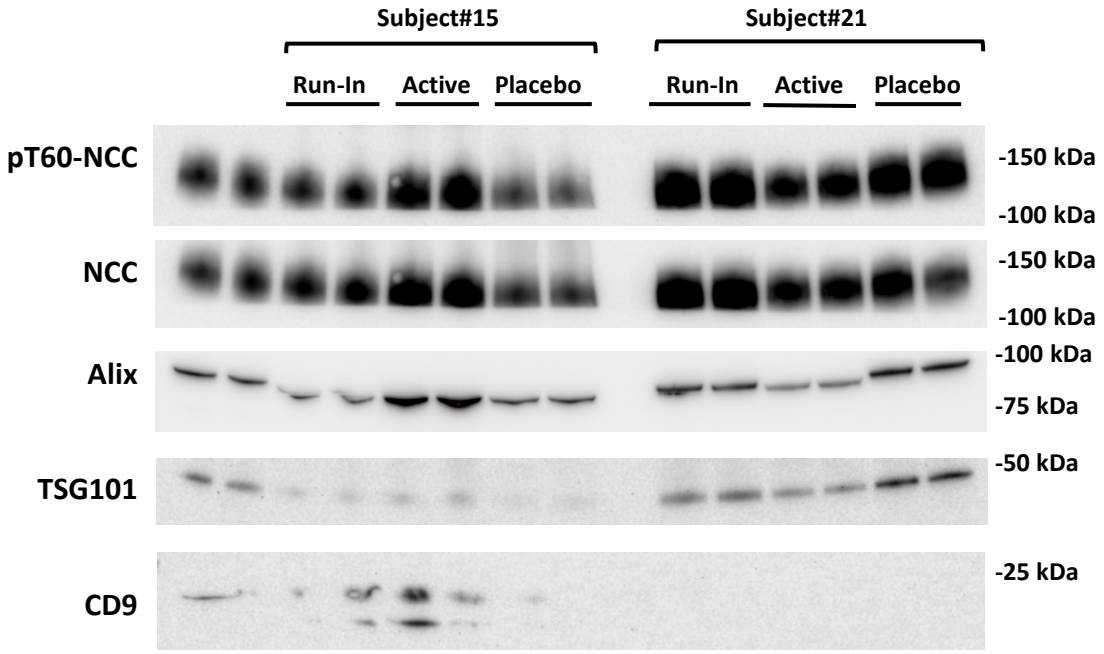
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-25 kDa

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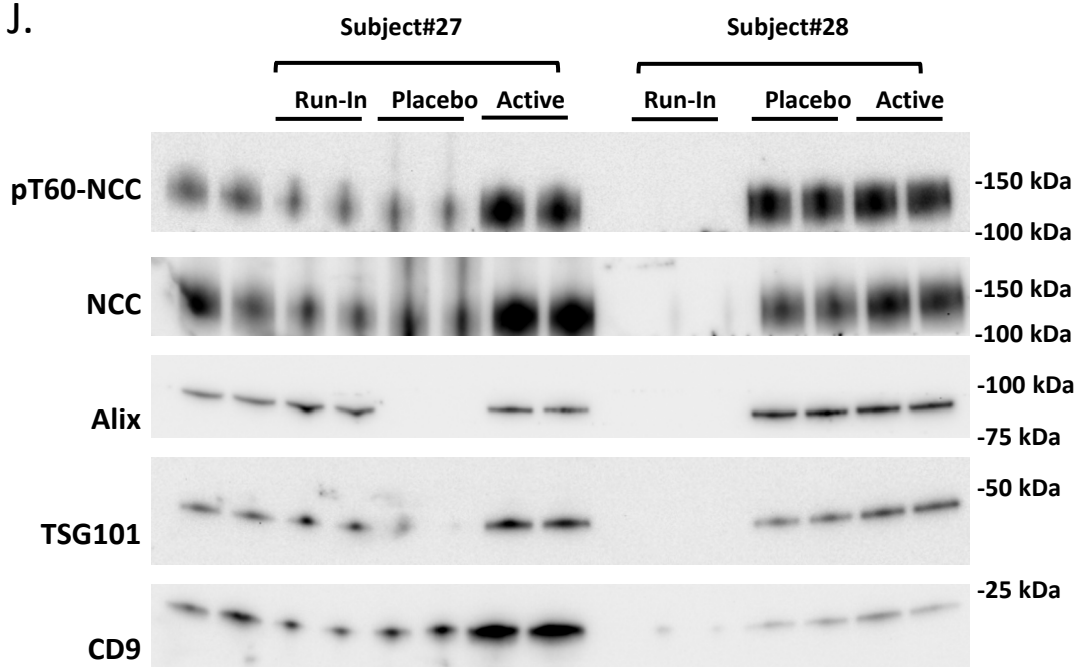
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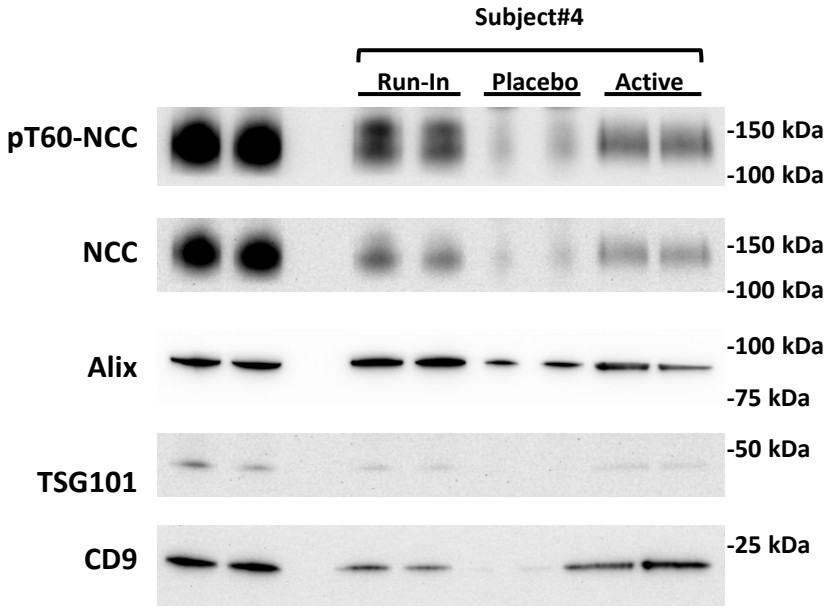
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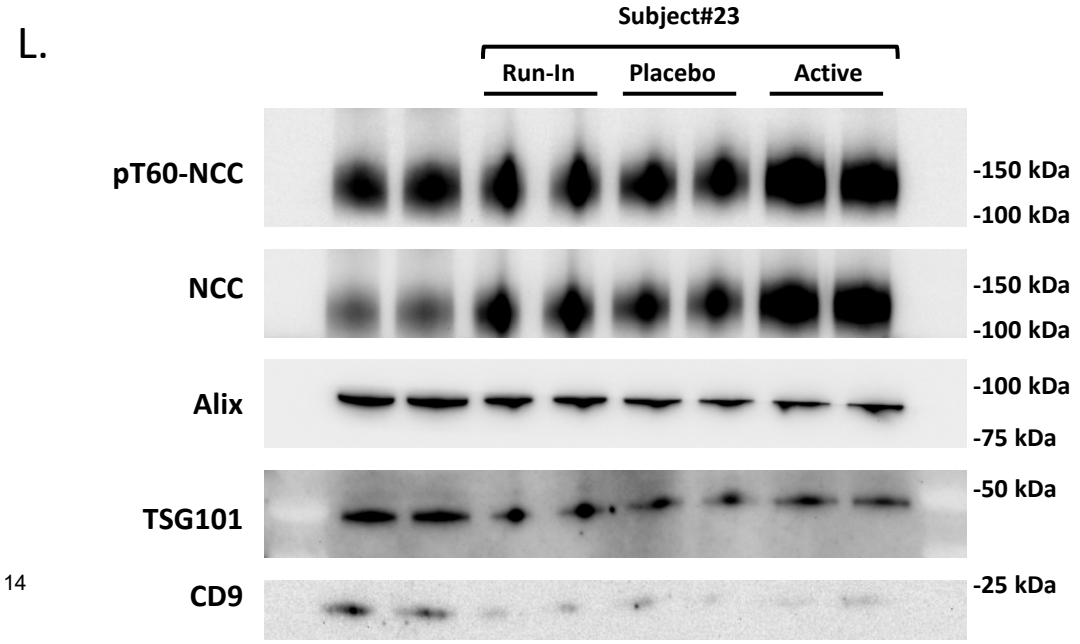
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-50 kDa
-25 kDa

Supplementary References

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