

Bulk RNA-seq Analysis Report

Report generated by Informagen Analytics



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Background

Bulk RNA-seq analysis of frozen primary head and neck squamous cell carcinoma (HNSC) tumors from C57BL/6J GEMM mice (25 independent biological samples) in a 2x2 factorial design comparing Keap1 status (wild type vs deleted) and BAP carcinogen exposure (present vs absent), encompassing simple contrasts, main effects, add-on effects, and the Keap1xBAP statistical interaction, to investigate how Keap1 loss and BAP treatment individually and interactively alter Nrf2 pathway activation, immune signaling, and related gene expression programs.

Study Design

- **Organism:** mouse
- **Environment:** in vivo
- **Sample preservation:** frozen
- **Samples:** 25

Variables recorded

- **keap1_status** — roles: primary; type: categorical; levels: Wt, Mut
- **bap_status** — roles: primary; type: categorical; levels: BAP, NoBAP

Biological Questions

- How do genes and pathways differ due to Keap1 status (wild type vs deleted) in tumors?
- How do genes and pathways differ due to BAP (carcinogen) treatment status (presence vs absence)?
- Does the effect of Keap1 status on genes/pathways differ depending on BAP treatment status (interaction)?
- Within each BAP status (yes/no), what genes and pathways differ due to Keap1 status (stratified analysis)?

Analysis Overview

- **Models:** categorical_deg
- **Analyses:** Differential Expression, GO Enrichment, GSEA, ssGSEA

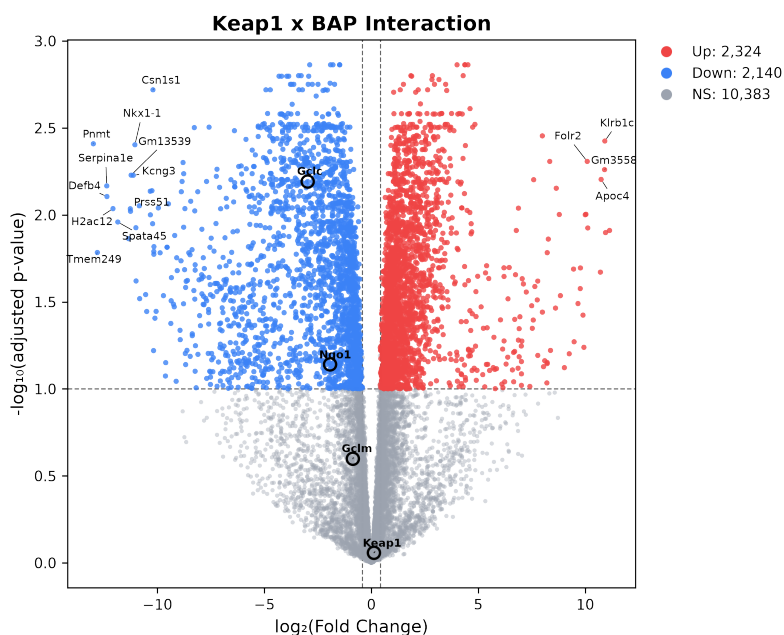
Results

How do genes and pathways differ due to BAP (carcinogen) treatment status (presence vs absence)?

Does the effect of Keap1 status on genes/pathways differ depending on BAP treatment status (interaction)?

Of 25 original primary mouse HNSCC tumors, 21 were retained after QC (Wt/NoBAP 5, Mut/NoBAP 4, Wt/BAP 5, Mut/BAP 7); all mice were C57BL/6J, with no reported batches. The fit $\sim 0 + \text{bap_status} + \text{keap1_status} + \text{gene_coverage}$ tests 14,847 genes across nine analysis labels representing seven distinct contrasts: two add-on labels duplicate matching simple effects and are not independent evidence. The verified fit includes one interaction. BAP-associated differential expression is extensive in Keap1-deleted tumors but sparse in wild-type tumors; sparse significance calls do not establish absent biological effects. At $\text{FDR} < 0.10$, the DEG interaction result contains 4,464 genes at $|\log_2\text{FC}| > 0.415$, whereas the response map contains 4,273 at the stricter $|\text{interaction}| > 0.585$ threshold. The map defines X as the BAP effect in wild-type tumors, Y as the BAP effect in deleted tumors, and the interaction as Y minus X. Opposite-signed fitted BAP responses account for 2,962 mapped genes, approximately 69%, supporting statistical interaction rather than established biological synergy or a specific mechanism. Interaction-associated biological-process results implicate extracellular matrix organization in the positive direction and keratinization in the negative direction. These processes should be distinguished from cell-surface and extracellular-matrix location annotations, which are GO cellular-component terms.

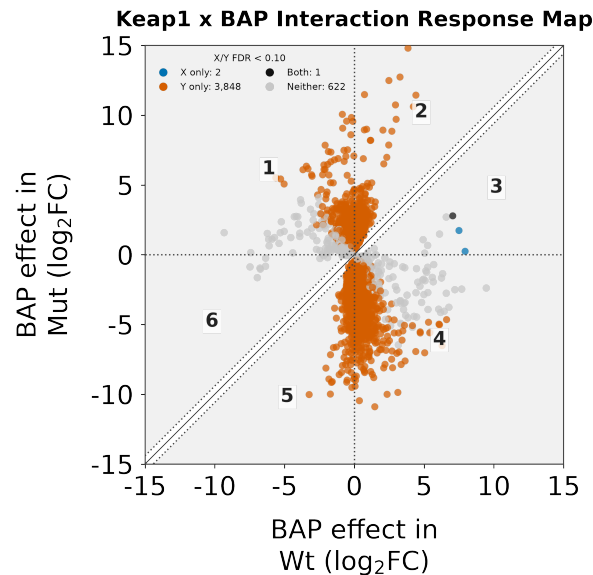
Differential Expression — Model: categorical_deg



Statistical interaction testing whether the effect of Keap1 status on gene expression differs depending on BAP treatment status. Thousands of genes show significant interaction effects, indicating the combined treatment's impact is not simply additive — described here as a statistical interaction rather than established biological synergy.

Response: bap_status, BAP versus NoBAP; context: keap1_status, Mut versus Wt. Fixed conditions: none. Interaction $I = Y - X$. Original region assignment uses interaction $\text{FDR} < 0.10$ and $|I| \geq 0.585$. region 1: 1423 original genes; region 2: 778 original genes; region 3: 11 original genes; region 4: 1539 original genes;

region 5: 510 original genes; region 6: 12 original genes. Layout: publication. Shared axes ± 15 ; 0 plotted genes shown at axis boundaries. Boundary placement does not change original values or region membership. Full original-gene and region exports remain independent of the rendered view.



Plots the fitted BAP treatment effect within Keap1-mutant tumors (Y) against the BAP treatment effect within Keap1-wild-type tumors (X); the interaction is defined as Y minus X. Genes are categorized into six expression-response patterns (e.g., reversal from increased to decreased expression, or the opposite reversal), describing observed response patterns rather than confirming a specific biological mechanism. All tested genes are shown in the background to illustrate the global distribution, with region membership based on the fitted interaction significance threshold ($FDR < 0.1$, $|\log_2FC| > 0.585$).

Summary

6,665 genes significant in ≥ 1 of 4 simple contrasts:

- **Effect of Keap1 status (Mutant vs Wild type) on tumors in the absence of BAP carcinogen treatment:**
 - 8 up (top: *Prl2c2*, *Marco*, *Ctxn3*, *Vsig4*, *Blvrb*; max $\log_2FC=16.12$)
- **Effect of BAP carcinogen treatment within Keap1 wild-type tumors: Wt_BAP vs Wt_NoBAP:**
 - 3 up (top: *Plin5*, *Pcdhgb6*, *Zfp105*; max $\log_2FC=7.94$)
- **Within Keap1-mutant tumors, effect of BAP carcinogen treatment versus no BAP treatment (Mut_BAP vs Mut_NoBAP):**
 - 3348 up (top: *Foxg1*, *Xcl1*, *Gbp2b*, *Apoc4*, *C7*; max $\log_2FC=14.80$);
 - 2683 down (top: *Serpina1e*, *Spink1*, *Slc22a7*, *Defb4*, *Slco1a7*; min $\log_2FC=-10.90$)
- **Effect of Keap1 status (Mutant vs Wild type) on gene/pathway expression within BAP-treated tumors (Mut_BAP_Group vs Wt_BAP_Group):**
 - 1581 up (top: *Prl2c2*, *Vsig4*, *Ighg2c*, *Nr1h5*, *Lyz1*; max $\log_2FC=17.36$);
 - 1693 down (top: *Defb4*, *Csn1s1*, *Nkx1-1*, *Sprr2a2*, *Bpifa5*; min $\log_2FC=-14.00$)
- **Main effect of Keap1 status (Mutant vs Wild type) on gene expression, pooled across BAP treatment status (averaging BAP and NoBAP conditions):**
 - 54 up (top: *Prl2c2*, *Vsig4*, *Ighg2c*, *Panx2*, *Radx*; max $\log_2FC=16.74$);

- 21 down (top: Csn1s1, Fabp4, Cbs, Chn2, Car2; min log2FC=-7.67)
- **Main effect of BAP carcinogen treatment status (BAP vs NoBAP), pooled across Keap1 status (Wt and Mut), comparing Wt_BAP_Group and Mut_BAP_Group combined versus Wt_NoBAP_Group and Mut_NoBAP_Group combined.:**
 - 1686 up (top: Foxg1, Gzmd, Xcl1, C7, Gbp2b; max log2FC=9.32);
 - 1108 down (top: Chil4, Gm28374, Slc22a7, Serpinb1c, Npw; min log2FC=-7.09)
- **Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.:**
 - 3348 up (top: Foxg1, Xcl1, Gbp2b, Apoc4, C7; max log2FC=14.80);
 - 2683 down (top: Serpina1e, Spink1, Slc22a7, Defb4, Slco1a7; min log2FC=-10.90)
- **Effect of Keap1 deletion (Mut vs Wt) in the presence of BAP carcinogen treatment, i.e. the add-on effect of Keap1 mutation status on top of BAP exposure.:**
 - 1581 up (top: Prl2c2, Vsiga4, Ighg2c, Nr1h5, Lyz1; max log2FC=17.36);
 - 1693 down (top: Defb4, Csn1s1, Nkx1-1, Sprr2a2, Bpifa5; min log2FC=-14.00)
- **Tests whether the effect of Keap1 status (Mut vs Wt) on tumor gene/pathway expression differs depending on BAP carcinogen treatment status, i.e., the interaction between Keap1 genotype and BAP exposure (synergy/antagonism beyond additive effects of each factor alone).:**
 - 2324 up (top: 1700001K19Rik, Foxg1, Klrk1c, Gm3558, Apoc4; max log2FC=11.14);
 - 2140 down (top: Pnmt, Tmem249, Serpina1e, Defb4, H2ac12; min log2FC=-12.99)

Top Up-Regulated Genes

Gene	log2FC	FDR	Contrast
Foxg1	14.800	1.68e-04	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Xcl1	12.731	3.27e-04	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Gbp2b	12.482	1.99e-04	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Apoc4	11.475	2.65e-04	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
C7	11.434	6.41e-04	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Dnai3	10.734	3.52e-04	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Pwwp3b	10.609	5.08e-04	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Gzmd	10.296	2.05e-02	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Klrb1c	10.060	3.27e-04	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Zar1	9.831	1.00e-03	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.

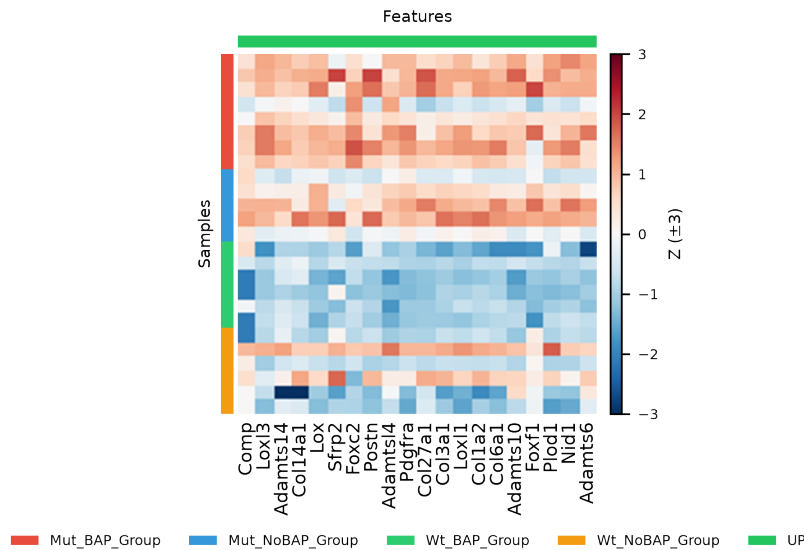
Top Down-Regulated Genes

Gene	log2FC	FDR	Contrast
Serpina1e	-10.897	1.21e-03	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Spink1	-10.463	5.70e-03	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Slc22a7	-10.025	1.02e-03	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Defb4	-10.006	2.60e-03	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Slco1a7	-9.990	4.68e-05	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Pnmt	-9.872	1.31e-03	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Gml	-9.518	2.66e-03	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Sprr2a2	-9.425	7.24e-04	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Csta3	-9.155	7.13e-03	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.
Traj58	-9.139	2.11e-03	Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.

GO Enrichment — Model: categorical_deg

extracellular matrix organization (GO:0030198); source list per_contrast_tier1_up_Synergy_Keap1_BAP. Original P value 2.2705e-11; FDR 1.11442e-08; fold enrichment 3.88076; 33 mapped study genes of 493; background membership 252 of 14610. Selected through: significant at original FDR <0.1. Display: 20 of 33 original source rows, showing per-sample expression z scores. Figure caps do not change complete gene/workbook exports. Enrichment and these display patterns do not establish pathway activation or causality.

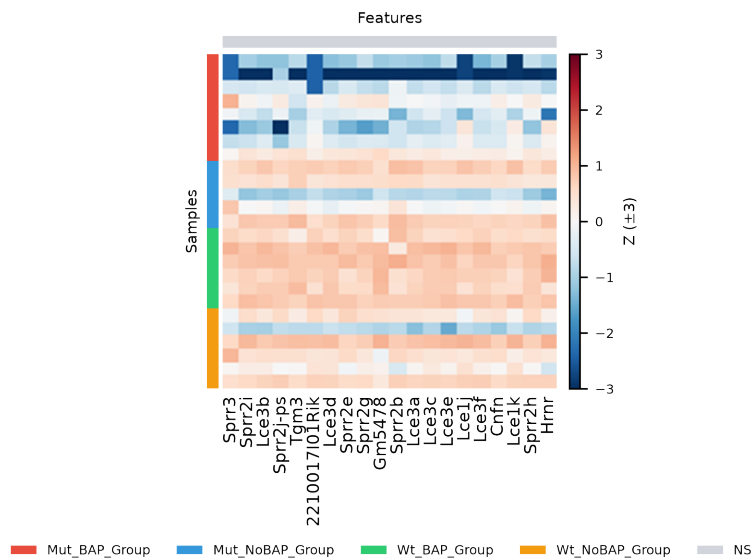
Extracellular matrix organization
Significant GO enrichment
20 of 33 source genes; sample expression.



Expression of up to 20 contributing genes for extracellular matrix organization (GO:0030198), enriched in the tier-1 positive interaction-contrast list. Samples show the four tumor groups; the heatmap does not establish a biological mechanism.

keratinization (GO:0031424); source list per_contrast_tier1_down_Synergy_Keap1_BAP. Original P value 4.27858e-50; FDR 4.83009e-46; fold enrichment 18.2368; 47 mapped study genes of 489; background membership 77 of 14610. Selected through: significant at original FDR <0.1. Display: 20 of 47 original source rows, showing per-sample expression z scores. Figure caps do not change complete gene/workbook exports. Enrichment and these display patterns do not establish pathway activation or causality.

Keratinization
Significant GO enrichment
20 of 47 source genes; sample expression.



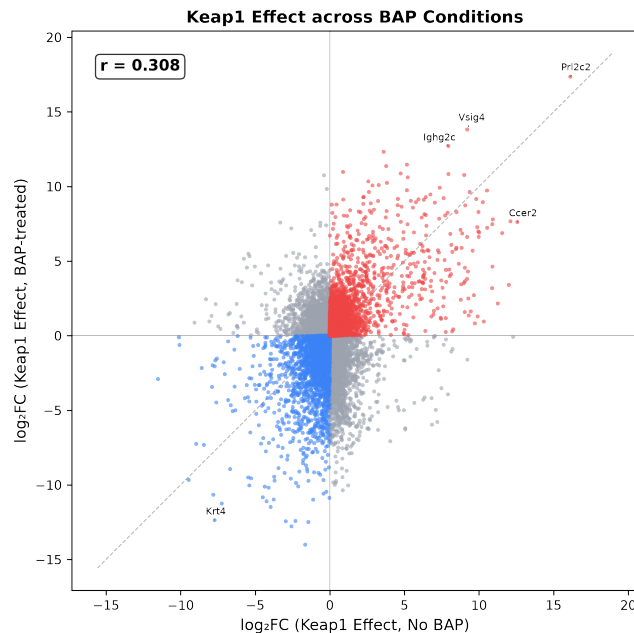
Expression of up to 20 contributing genes for keratinization (GO:0031424), enriched in the tier-1 negative interaction-contrast list. Samples show the four tumor groups; the heatmap does not establish a biological mechanism.

How do genes and pathways differ due to Keap1 status (wild type vs deleted) in tumors?

Within each BAP status (yes/no), what genes and pathways differ due to Keap1 status (stratified analysis)?

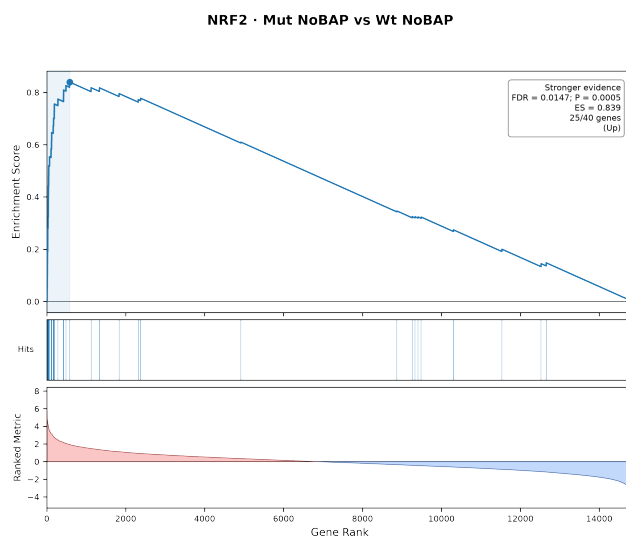
Keap1-associated differential expression is much more extensive under BAP than without BAP, but few significant baseline DEGs do not demonstrate minimal biological or transcriptomic impact. Without BAP, the comparison of 4 Keap1-deleted versus 5 wild-type tumors shows positive NRF2 GSEA enrichment (rotation $P=0.0005$; $FDR=0.0146667$) and a higher NRF2 ssGSEA score in deleted tumors, with a score difference of +1131.45 (rotation $P=0.002$; $FDR=0.044$). The ssGSEA difference is in score units, not log2 fold change. Together, enrichment, its contributing leading-edge genes, and sample-level scoring support an NRF2-associated expression program despite sparse baseline gene-level significance calls. This baseline finding must be distinguished from the genome-wide interaction: the NRF2 ssGSEA interaction is not significant ($FDR=0.2003$), so a significant NRF2-specific interaction is not established. These conclusions concern gene-expression signatures, not directly measured NRF2 protein activity or immune-cell abundance.

Differential Expression — Model: categorical_deg

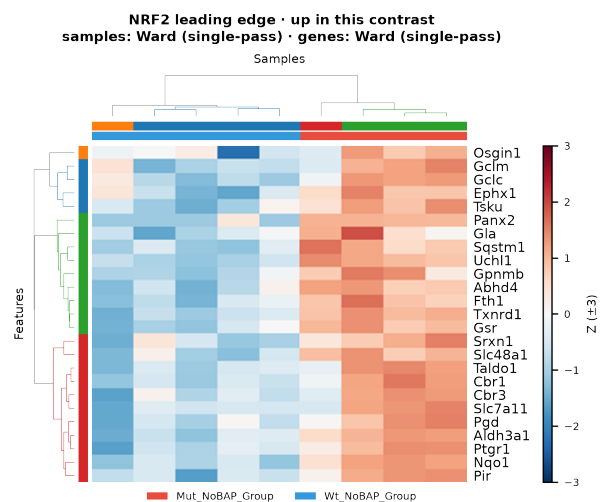


Scatter of Keap1 mutation effect (Mut vs Wt) in the absence of BAP versus the same Keap1 effect within BAP-treated tumors. Convergence coloring highlights genes with consistent versus divergent Keap1-driven responses depending on carcinogen exposure.

GSEA — Model: categorical_deg



GSEA enrichment plot for NRF2 in Effect of Keap1 status (Mutant vs Wild type) on tumors in the absence of BAP carcinogen treatment



Per-sample expression of the leading-edge genes driving NRF2 in Effect of Keap1 status (Mutant vs Wild type) on tumors in the absence of BAP carcinogen treatment.

Summary

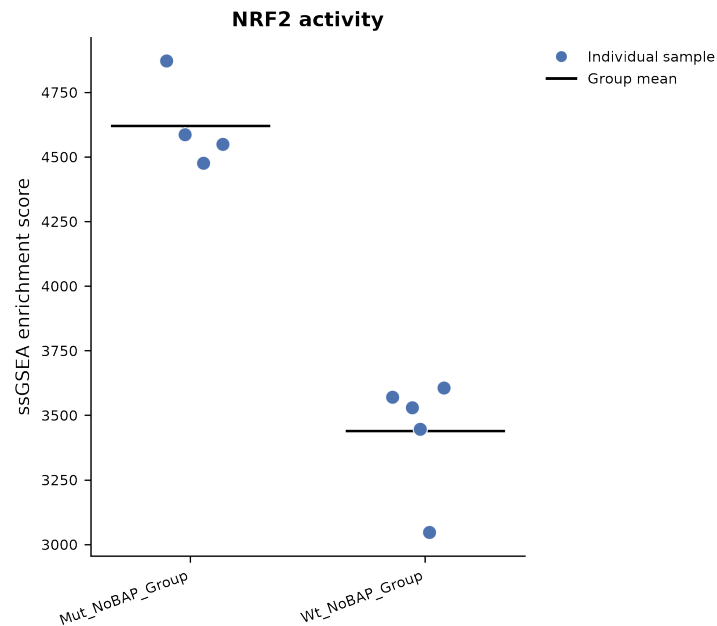
GSEA tested the complete frozen set of **88 pathways** for **9 inference target(s)** using 1,999 residual rotations per target. Rotation P values are plus-one empirical tails and rotation FDR is Benjamini-Hochberg corrected within each target.

Evidence bands are descriptive renderings of continuous rotation FDR; they are not biological verdicts or composition-based validation gates.

Target	Pathways	FDR < 0.05	FDR < 0.10	FDR < 0.15	Minimum FDR
Effect of Keap1 status (Mutant vs Wild type) on tumors in the absence of BAP carcinogen treatment	88	4	4	4	0.01467
Effect of BAP carcinogen treatment within Keap1 wild-type tumors: Wt_BAP vs Wt_NoBAP	88	0	0	0	0.7901
Within Keap1-mutant tumors, effect of BAP carcinogen treatment versus no BAP treatment (Mut_BAP vs Mut_NoBAP)	88	0	1	1	0.088
Effect of Keap1 status (Mutant vs Wild type) on gene/pathway expression within BAP-treated tumors (Mut_BAP_Group vs Wt_BAP_Group)	88	0	0	0	0.176
Main effect of Keap1 status (Mutant vs Wild type) on gene expression, pooled across BAP treatment status (averaging BAP and NoBAP conditions).	88	0	0	0	0.5192
Main effect of BAP carcinogen treatment status (BAP vs NoBAP), pooled across Keap1 status (Wt and Mut), comparing Wt_BAP_Group and Mut_BAP_Group combined versus Wt_NoBAP_Group and Mut_NoBAP_Group combined.	88	0	0	0	0.8421
Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.	88	0	0	0	0.396
Effect of Keap1 deletion (Mut vs Wt) in the presence of BAP carcinogen treatment, i.e. the add-on effect of Keap1 mutation status on top of BAP exposure.	88	0	0	1	0.132
Tests whether the effect of Keap1 status (Mut vs Wt) on tumor gene/pathway expression differs depending on BAP carcinogen treatment status, i.e., the interaction between Keap1 genotype and BAP exposure (synergy/antagonism beyond additive effects of each factor alone).	88	0	0	0	0.3872

Lowest rotation-FDR results

Target	Pathway	Effect	Rotation P	Rotation FDR	Evidence band
Effect of Keap1 status (Mutant vs Wild type) on tumors in the absence of BAP carcinogen treatment	NRF2	0.8392	0.0005	0.01467	Stronger evidence
Effect of Keap1 status (Mutant vs Wild type) on tumors in the absence of BAP carcinogen treatment	HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY	0.6808	0.0005	0.01467	Stronger evidence
Effect of Keap1 status (Mutant vs Wild type) on tumors in the absence of BAP carcinogen treatment	HALLMARK_XENOBIOTIC_METABOLISM	0.5175	0.0005	0.01467	Stronger evidence
Effect of Keap1 status (Mutant vs Wild type) on tumors in the absence of BAP carcinogen treatment	HALLMARK_HEME_METABOLISM	0.4195	0.0015	0.033	Stronger evidence
Within Keap1-mutant tumors, effect of BAP carcinogen treatment versus no BAP treatment (Mut_BAP vs Mut_NoBAP)	HALLMARK_CHOLESTEROL_HOMEOSTASIS	-0.556	0.001	0.088	Moderate evidence
Effect of Keap1 deletion (Mut vs Wt) in the presence of BAP carcinogen treatment, i.e. the add-on effect of Keap1 mutation status on top of BAP exposure.	MoDC	0.8715	0.0015	0.132	Borderline/threshold-adjacent
Effect of Keap1 deletion (Mut vs Wt) in the presence of BAP carcinogen treatment, i.e. the add-on effect of Keap1 mutation status on top of BAP exposure.	HALLMARK_KRAS_SIGNALING_DN	-0.4666	0.0035	0.154	Little statistical support
Effect of Keap1 status (Mutant vs Wild type) on tumors in the absence of BAP carcinogen treatment	HALLMARK_BILE_ACID_METABOLISM	0.4056	0.009	0.1584	Little statistical support
Effect of Keap1 status (Mutant vs Wild type) on gene/pathway expression within BAP-treated tumors (Mut_BAP_Group vs Wt_BAP_Group)	MoDC	0.8715	0.004	0.176	Little statistical support
Effect of Keap1 status (Mutant vs Wild type) on gene/pathway expression within BAP-treated tumors (Mut_BAP_Group vs Wt_BAP_Group)	HALLMARK_KRAS_SIGNALING_DN	-0.4666	0.003	0.176	Little statistical support

ssGSEA — Model: categorical_deg

Per-sample ssGSEA score for NRF2 across Mut_NoBAP_vs_Wt_NoBAP.

Summary

ssGSEA tested the complete frozen set of **88 pathways** for **9 inference target(s)** across **21 samples** using 1,999 residual rotations per target. Rotation P values are plus-one empirical tails and rotation FDR is Benjamini-Hochberg corrected within each target.

Evidence bands are descriptive renderings of continuous rotation FDR; they are not biological verdicts or composition-based validation gates.

Target	Pathways	FDR < 0.05	FDR < 0.10	FDR < 0.15	Minimum FDR
Effect of Keap1 status (Mutant vs Wild type) on tumors in the absence of BAP carcinogen treatment	88	4	7	11	0.044
Effect of BAP carcinogen treatment within Keap1 wild-type tumors: Wt_BAP vs Wt_NoBAP	88	0	0	0	0.6974
Within Keap1-mutant tumors, effect of BAP carcinogen treatment versus no BAP treatment (Mut_BAP vs Mut_NoBAP)	88	31	42	48	0.0044
Effect of Keap1 status (Mutant vs Wild type) on gene/pathway expression within BAP-treated tumors (Mut_BAP_Group vs Wt_BAP_Group)	88	14	30	33	0.022
Main effect of Keap1 status (Mutant vs Wild type) on gene expression, pooled across BAP treatment status (averaging BAP and NoBAP conditions).	88	0	2	5	0.066
Main effect of BAP carcinogen treatment status (BAP vs NoBAP), pooled across Keap1 status (Wt and Mut), comparing Wt_BAP_Group and Mut_BAP_Group combined versus Wt_NoBAP_Group and Mut_NoBAP_Group combined.	88	1	5	12	0.044
Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.	88	33	44	47	0.003385
Effect of Keap1 deletion (Mut vs Wt) in the presence of BAP carcinogen treatment, i.e. the add-on effect of Keap1 mutation status on top of BAP exposure.	88	16	30	33	0.0264
Tests whether the effect of Keap1 status (Mut vs Wt) on tumor gene/pathway expression differs depending on BAP carcinogen treatment status, i.e., the interaction between Keap1 genotype and BAP exposure (synergy/antagonism beyond additive effects of each factor alone).	88	12	24	26	0.0088

Lowest rotation-FDR results

Target	Pathway	Effect	Rotation P	Rotation FDR	Evidence band
Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.	Memory_B	1221	0.0005	0.003385	Stronger evidence
Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.	HALLMARK_UV_RESPONSE_DN	801.8	0.0005	0.003385	Stronger evidence
Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.	HALLMARK_KRAS_SIGNALING_DN	-771.4	0.0005	0.003385	Stronger evidence
Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.	HALLMARK_E2F_TARGETS	688.4	0.0005	0.003385	Stronger evidence
Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.	HALLMARK_G2M_CHECKPOINT	575.4	0.0005	0.003385	Stronger evidence
Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.	HALLMARK_MITOTIC_SPINDLE	555.5	0.0005	0.003385	Stronger evidence

Target	Pathway	Effect	Rotation P	Rotation FDR	Evidence band
Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.	HALLMARK_PANCREAS_BETA_CELLS	478	0.0005	0.003385	Stronger evidence
Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.	HALLMARK_BILE_ACID_METABOLISM	-381	0.0005	0.003385	Stronger evidence
Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.	HALLMARK_UNFOLDED_PROTEIN_RESPONSE	362.6	0.0005	0.003385	Stronger evidence
Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.	HALLMARK_APOPTOSIS	301.3	0.0005	0.003385	Stronger evidence

Discussion

The interaction between Keap1 status and BAP carcinogen exposure emerges as the most transcriptionally consequential axis of this 2×2 experiment, though it must be framed carefully as a statistical rather than a demonstrated biological phenomenon. At FDR<0.10 the interaction DEG result contains 4,464 genes passing $|\log_2\text{FC}| > 0.415$, and the response map retains 4,273 genes at the stricter $|\log_2\text{FC}| > 0.585$ threshold. Of these mapped genes, approximately 69% (2,962) show opposite-signed fitted BAP responses in wild-type versus Keap1-deleted tumors, meaning the direction of the carcinogen response is itself contingent on genotype rather than merely its magnitude. This pattern is internally consistent with the simple-contrast summaries: BAP exposure produces 3,348 up- and 2,683 down-regulated genes within Keap1-mutant tumors, but only three up-regulated genes within wild-type tumors. The extensive divergence drives the interaction signal. However, the Synergy_Keap1_BAP comparison carries the lowest effective sample size in the design (≈ 3.0 per group), so despite the large gene counts the estimate rests on the thinnest replication available and should be read as hypothesis-generating. The interaction GO results implicate extracellular matrix organization in the positive direction (GO:0030198; FDR 1.11e-08, fold enrichment 3.88) and keratinization in the negative direction (GO:0031424; FDR 4.83e-46, fold enrichment 18.2). Keratinization enrichment in the negative interaction list describes a genotype-dependent difference in the BAP response. It does not by itself establish absolute suppression in the combined group, dedifferentiation, or stromal remodeling; these are hypotheses for orthogonal validation. These are biological-process terms and should not be conflated with cellular-component location annotations.

Within Keap1-mutant tumors the BAP add-on contrast duplicates the Mut_BAP_vs_Mut_NoBAP simple effect and therefore does not provide independent evidence; the shared top genes (Foxg1, Xcl1, Gbp2b, Apoc4, C7 up; Serpina1e, Spink1, Slc22a7, Defb4, Slco1a7 down) reflect a single underlying comparison. The up-regulated leading genes include several immune and interferon-associated transcripts (Xcl1, Gbp2b, C7, Klr1c, Gzmd), consistent with an inflammatory response to carcinogen exposure that is only unmasked on the Keap1-deleted background, while the down-regulated set is dominated by secreted protease inhibitors and epithelial/keratinization-associated genes (Serpina1e, Spink1, Defb4, Sprr2a2, Csta3), echoing the keratinization suppression seen in the GO analysis. The ssGSEA results for this contrast reinforce a proliferative and stress-response shift, with strong positive scores for HALLMARK_E2F_TARGETS, HALLMARK_G2M_CHECKPOINT, and HALLMARK_MITOTIC_SPINDLE and negative scores for HALLMARK_KRAS_SIGNALING_DN and HALLMARK_BILE_ACID_METABOLISM, all in the stronger-evidence band of the continuous rotation-FDR rendering (FDR $\approx$ 0.0034). In the GSEA of the same mutant-BAP contrast, HALLMARK_CHOLESTEROL_HOMEOSTASIS appears with a negative effect in the moderate-evidence band (FDR 0.088), indicating that the gene-level rank signal is weaker than the sample-level enrichment for this comparison.

The Keap1-status question is best resolved in the no-BAP baseline stratum, where gene-level significance is sparse (8 up-regulated genes, top Prl2c2, Marco, Ctxn3, Vsig4, Blvrb) but the pathway evidence is coherent. NRF2 shows positive GSEA enrichment (effect 0.8392; rotation P=0.0005; FDR 0.0147) in the stronger-evidence band, accompanied in the same contrast by HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY and HALLMARK_XENOBIOTIC_METABOLISM (both FDR 0.0147) and HALLMARK_HEME_METABOLISM (FDR 0.033). This is the canonical signature expected from Keap1 loss, which stabilizes NRF2 and activates antioxidant and xenobiotic-metabolism transcriptional programs. The ssGSEA result supports the same direction at the sample level: NRF2 scores are higher in deleted tumors with a difference of +1131.45 score units (rotation P=0.002; FDR 0.044), also

in the stronger-evidence band. Because the GSEA and ssGSEA both report NRF2 for this same contrast, their concordant positive direction can be described side by side as a descriptive agreement; this is not a formal concordance test and the two effect magnitudes are not on a shared scale. Critically, this NRF2 signal is a baseline feature: the NRF2 ssGSEA interaction is not significant (FDR 0.2003), so there is no established evidence that Keap1-driven NRF2 activation is itself modified by BAP, even though the genome-wide interaction is extensive. The Marco and Vsig4 genes among the baseline Keap1 DEGs are macrophage markers, hinting that Keap1 loss may also shift myeloid composition, though these are expression signatures rather than directly measured cell abundances. Keap1-status effects were far more extensive within BAP-treated tumors (1,581 up, 1,693 down) than at baseline, consistent with carcinogen exposure amplifying genotype-dependent divergence; the in-BAP and Keap1-in-BAP add-on labels represent the same mathematical contrast. No GSEA pathway in that comparison meets FDR <0.10; ssGSEA findings must be attributed to ssGSEA and are not independent evidence from the duplicate label.

All quantitative interpretations must be weighted against the diagnostic context. The model diagnostics — residual degrees of freedom, parameter-to-sample ratio, effective sample sizes, and power warnings — were recorded during model selection and describe the planning cohort, not the executed fit. The analysis was executed on 21 retained samples after QC removed four of the original 25, distributed unevenly across the four groups (Wt/NoBAP 5, Mut/NoBAP 4, Wt/BAP 5, Mut/BAP 7). The planning-stage effective-N estimates flag every comparison as underpowered relative to the target of 15 per group, with the interaction being the most extreme; these should be treated as stage-qualified planning values rather than recomputed post-QC fit diagnostics. The practical consequence is that the large DEG counts reflect sensitivity to large effects and the many near-reversal genes should be regarded as candidates for confirmation rather than settled findings. No Cross-Analysis Findings section was supplied, and no formal cross-comparison overlap, DEG-GSEA convergence, or DEG-GO concordance test was performed; such concordance was therefore not assessed beyond the single same-contrast NRF2 comparison noted above.

Several follow-up directions emerge naturally from these patterns. The foremost question is whether the genotype-dependent reversal of the BAP response is reproducible in a cohort balanced and powered for the interaction, given the small retained groups and limited sensitivity flagged by planning-stage diagnostics; a dedicated replication with balanced, expanded groups would directly test the 69% opposite-sign observation. Orthogonal validation of the NRF2 axis is warranted at the protein level, measuring nuclear NRF2 stabilization and canonical targets (for example NQO1 and GCLC) to confirm that the baseline transcriptional signature reflects genuine pathway activation rather than a correlated expression program. The immune and myeloid signals — Xcl1, Gbp2b, and complement component C7 induction alongside baseline Marco and Vsig4 — raise the hypothesis that Keap1 loss reshapes the tumor immune microenvironment, which could be tested by flow cytometry or immunohistochemistry to resolve whether the signatures reflect true changes in immune-cell abundance, since the present data cannot distinguish composition from per-cell expression. The negative keratinization interaction and positive extracellular-matrix organization interaction suggest hypotheses that could be examined histologically and through stromal profiling. Neither establishes dedifferentiation or absolute pathway suppression in the combined group. Finally, because the mutant-BAP contrast shows coordinated activation of E2F, G2M, and mitotic-spindle programs, a functional test of whether Keap1 loss sensitizes carcinogen-exposed tumors to proliferative or antioxidant-pathway-directed interventions would convert these descriptive signatures into a testable mechanistic model.

Methods and Model Diagnostics

This appendix documents the statistical models, design diagnostics, power indicators, and run parameters used for the reported results.

Diagnostic provenance: Samples used refers to the retained execution cohort after QC. The model and comparison diagnostics below were recorded during model selection; they are not recomputed diagnostics of the executed fit. If QC changed the cohort, their residual degrees of freedom, parameter-to-sample ratio, effective sample size, and power assessments may describe the earlier planning cohort and must not be treated as post-QC fit estimates.

Analysis Models

categorical_deg

Diagnostic	Value
Model type	categorical_deg
Design formula	~ 0 + bap_status:keap1_status + gene_coverage
Samples used	21
Design rank / estimated parameters	5
Residual degrees of freedom	20
Parameter-to-sample ratio	0.2
Batch model	none
Implicit covariates	gene_coverage
Dropped covariates	None
Unadjusted confounding override	No
Stability status	ok
Stability assessment	Design is sound.

Active model diagnostics

None

Applied model fixes

None

Comparison diagnostics

Comparison	Type	Feasible	Estimable	Outcome	Effective N	VIF	Batch relationship
Mut NoBAP vs Wt NoBAP	simple contrast	Yes	Yes	A	5.454545454 545454	1.0	crossed balanced
Wt BAP vs Wt NoBAP	simple contrast	Yes	Yes	A	6.0	1.0	crossed balanced
Mut BAP vs Mut NoBAP	simple contrast	Yes	Yes	A	6.153846153 846153	1.0	crossed balanced
Keap1Mut vs Wt in BAP	simple contrast	Yes	Yes	A	6.857142857 142858	1.0	crossed balanced
Main Keap1Mut vs Keap1Wt	main effect	Yes	Yes	A	12.15189873 4177216	1.0	crossed balanced
Main BAP vs NoBAP	main effect	Yes	Yes	A	12.15189873 4177218	1.0	crossed balanced
AddOn BAP to Keap1Mut	add on effect	Yes	Yes	A	6.153846153 846153	1.0	crossed balanced
AddOn Keap1Mut to BAP	add on effect	Yes	Yes	A	6.857142857 142858	1.0	crossed balanced
Synergy Keap1 BAP	synergy	Yes	Yes	A	3.037974683 544304	1.0	crossed balanced

Warnings affecting this model's comparisons

- **Limited statistical power (LOW_POWER)** — Affects 3 of 9 comparisons in this model. Target: keap1_status. The affected comparisons have limited sensitivity for smaller effects.
- **Limited statistical power (LOW_POWER)** — Affects 3 of 9 comparisons in this model. Target: bap_status. The affected comparisons have limited sensitivity for smaller effects.
- **Limited statistical power (LOW_POWER)** — Affects 3 of 9 comparisons in this model. The affected comparisons have limited sensitivity for smaller effects.

Task-Specific Methods

Differential Expression — Model: categorical_deg

Run details: Method: Limma | Genes tested: 14,847 | FDR: 0.1 | ** | log2FC | :** 0.415

Methodology Notes

Design matrix:

- **Gene-coverage covariate adjustment:** included. User confirmed standard QC sample weighting should apply; quality itself is not a primary research question, proceeding with normal post-omics weighting and gene coverage covariate adjustment.

Per-contrast caveats:

- **Add-on effect of BAP carcinogen treatment within Keap1-mutant tumors: comparing Keap1-mutant tumors with BAP to Keap1-mutant tumors without BAP, isolating the incremental impact of BAP exposure on top of Keap1 deletion.:**

- **LOW_POWER (unfixable):** Comparison 'AddOn_BAP_to_Keap1Mut' is estimable but underpowered: effective N per group ≈ 6.2 , below the target of 15 (detects only large effects).
- **Effect of BAP carcinogen treatment within Keap1 wild-type tumors: Wt_BAP vs Wt_NoBAP:**
- **LOW_POWER (unfixable):** Comparison 'Wt_BAP_vs_Wt_NoBAP' is estimable but underpowered: effective N per group ≈ 6.0 , below the target of 15 (detects only large effects).
- **Effect of Keap1 deletion (Mut vs Wt) in the presence of BAP carcinogen treatment, i.e. the add-on effect of Keap1 mutation status on top of BAP exposure.:**
- **LOW_POWER (unfixable):** Comparison 'AddOn_Keap1Mut_to_BAP' is estimable but underpowered: effective N per group ≈ 6.9 , below the target of 15 (detects only large effects).
- **Effect of Keap1 status (Mutant vs Wild type) on gene/pathway expression within BAP-treated tumors (Mut_BAP_Group vs Wt_BAP_Group):**
- **LOW_POWER (unfixable):** Comparison 'Keap1Mut_vs_Wt_in_BAP' is estimable but underpowered: effective N per group ≈ 6.9 , below the target of 15 (detects only large effects).
- **Effect of Keap1 status (Mutant vs Wild type) on tumors in the absence of BAP carcinogen treatment:**
- **LOW_POWER (unfixable):** Comparison 'Mut_NoBAP_vs_Wt_NoBAP' is estimable but underpowered: effective N per group ≈ 5.5 , below the target of 15 (detects only large effects).
- **Main effect of BAP carcinogen treatment status (BAP vs NoBAP), pooled across Keap1 status (Wt and Mut), comparing Wt_BAP_Group and Mut_BAP_Group combined versus Wt_NoBAP_Group and Mut_NoBAP_Group combined.:**
- **LOW_POWER (unfixable):** Comparison 'Main_BAP_vs_NoBAP' is estimable but underpowered: effective N per group ≈ 12.2 , below the target of 15 (detects only large effects).
- **Main effect of Keap1 status (Mutant vs Wild type) on gene expression, pooled across BAP treatment status (averaging BAP and NoBAP conditions).:**
- **LOW_POWER (unfixable):** Comparison 'Main_Keap1Mut_vs_Keap1Wt' is estimable but underpowered: effective N per group ≈ 12.2 , below the target of 15 (detects only large effects).
- **Tests whether the effect of Keap1 status (Mut vs Wt) on tumor gene/pathway expression differs depending on BAP carcinogen treatment status, i.e., the interaction between Keap1 genotype and BAP exposure (synergy/antagonism beyond additive effects of each factor alone).:**
- **LOW_POWER (unfixable):** Comparison 'Synergy_Keap1_BAP' is estimable but underpowered: effective N per group ≈ 3.0 , below the target of 15 (detects only large effects).
- **Within Keap1-mutant tumors, effect of BAP carcinogen treatment versus no BAP treatment (Mut_BAP vs Mut_NoBAP):**
- **LOW_POWER (unfixable):** Comparison 'Mut_BAP_vs_Mut_NoBAP' is estimable but underpowered: effective N per group ≈ 6.2 , below the target of 15 (detects only large effects).

Analysis Parameters

Parameter	Value
Batch Model	none
Method	limma
Contrast types	4 simple, 2 main effect, 2 add on effect, 1 interaction

Glossary

Differential Expression (DEG)

- **Positive log2FC** = up-regulated in treatment vs control; **negative** = down-regulated
- **linearFC** = $2^{(\log_2\text{FC})}$, the fold change on linear scale (e.g., 2.0 = 2x increase)
- **Simple contrasts** compare treatment vs control within one blocking unit
- **Interaction contrasts** test whether the treatment effect differs between blocking units — a significant gene responds differently to treatment depending on biological context
- **Universal genes** (significant in all simple contrasts, same direction) are the most robust responders
- **Supergroup convergence** genes are significant within a phenotype cluster but not necessarily universal

Gene Set Enrichment (GSEA)

- GSEA tests coordinated ranked-gene shifts; **positive GSEA ES** indicates enrichment toward the non-reference group and **negative GSEA ES** toward the reference group
- **Rotation FDR** quantifies statistical evidence from residual rotations while preserving the fitted design and nuisance adjustment
- **Leading-edge genes** are the subset most responsible for the observed pathway pattern
- Evidence bands summarize the continuous rotation FDR and are descriptive, not biological verdicts
- **GO overrepresentation** uses the explicitly prepared source list and its tested background. Ranked pathway tests address a different question; agreement alone does not establish activation or causality

Single-Sample Enrichment (ssGSEA)

- **Positive observed effect** = higher sample-level pathway scores in treatment vs control; **negative** = lower; larger absolute values indicate larger adjusted shifts
- **Rotation FDR** provides the pathway-level uncertainty measure; evidence bands are descriptive ranges of that continuous value
- ssGSEA calculates a pathway activity score for each sample, enabling sample-level comparisons
- **Per-sample scores** are useful for sample clustering, correlating pathway activity with phenotypes, or identifying outlier samples
- **Interaction effects**: pathways significant in interaction contrasts show differential regulation — the treatment effect on pathway activity differs between experimental units

GO Enrichment

- **Significant terms** meet the source analysis's original FDR cutoff. Exploratory views require fold enrichment ≥ 1.5 and their saved study-count minimum; selection is not proof of biological importance.
- **Ordinary GO heatmaps** show sample expression z scores; **interaction GO heatmaps** show fitted Y, X and I log2 fold changes with $I = Y - X$.
- **Display limits** affect figures only. Full tests, gene membership and automatic significant/exploratory workbooks retain all source rows.

- **Fold enrichment:** how many times more genes than expected by chance (2x = twice as many). Raw enrichment is nonnegative; overrepresentation is tested against the mapped fitted-gene universe
- **Counting scope:** counts refer to term-list pairs. Overlapping categories are complementary views, not independent discoveries
- **GO domains:** BP (Biological Process) = what genes do; MF (Molecular Function) = biochemical activity; CC (Cellular Component) = subcellular location
- **Universal enrichment:** GO terms enriched in `universal_up`/`universal_down` lists are consistently affected across all conditions
- **Supergroup enrichment:** GO terms in `supergroup_*` lists are shared by convergent phenotype groups
- **Per-contrast enrichment:** GO terms in individual contrast lists represent condition-specific effects