Supplemental Tables and Figures
Burel et al. 2015

Supplemental Table 1: Description of RT-PCR primers and probes.

Supplemental Table 2: Number of perfect matches and partial matches in the liver pre mRNA transcriptome for each LNA ASOs. Columns label 0 to 9 represent the number of mismatches .per putative ASO:RNA duplexes.

Supplemental Figure1: Weblogo representation of the sequence space of LNA ASOs: Positional frequency of nucleotide residues in gap aligned LNA ASOs. (A) All ASO (B) Well tolerated ASOs, (C) Toxic ASOs.

Supplemental Figure 2: Liver histopathology from mice treated with either saline or hepatotoxic LNA ASO ISIS 569720 (FXI-2) dosed with 11, 33 and 100 mg/kg, 96 hours after s.c. administration. Hematoxylin and eosin staining (2000x).

Supplemental Figure 3: The effects of 3 different type of nucleotide modifications (cEt, LNA and MOE) on the increase of ALT as well as on and off target knockdown were compared for 3 hepatotoxic LNA ASO sequences. (A, top panel) Plasma ALT levels from of Balb/c mice (n=4 per dose) treated with increasing doses of ASOs were measured at 72 hours. (A, bottom 3 panels) On and Off Target mRNA reduction was assessed 24 hours post treatment in the liver by RT-PCR. RNA reduction are presented as percentage reduction relative to saline treated animals. (B) Comparing on target mRNA reduction (solid blue line) for 2 ASO sequences with 3 different nucleotide modification (cEt, LNA and MOE) with 16 different off target mRNAs (dotted lines) 24 hours post treatment in the liver by RT-PCR. The mean reduction for the 16 off target mRNA is also represented (solid red line).

Supplemental Figure 4. Assessment of the canonical biological pathways enriched following treatment with LNA using Ingenuity Pathway Analysis Software. (A) Oneway hierarchical clustering of canonical pathways significantly enriched for each ASO administered at the maximum tolerated dose of 300 mg/kg or at a lowest dose producing at least 1000 IU/mL ALT increase at 96 hours. (B) List of modulated pathways based on 594 transcripts modulated at 24 hours in correlation with ALT increase at 96 hours. Standard deviation is shown.

Supplemental Figure 5. RNase H1 can only cleave ASO:RNA duplexes if a central portion of the ASO is unmodified. Addition of modified nucleotides to the central region (gap) should reduce or abrogate RNase H activity. (A) Two hepatotoxic cEt ASO were modified to include 3 cEt modifications (in red) in the gap. Balb/c mice (4/group) were treated by subcutaneous injection with a single dose of 200 mg/kg (PTEN) or 50 mg/kg (FVII) gap unmodified or modified ASO. (BC) Liver on-target mRNA levels (FVII and PTEN) (B) and plasma ALT levels (C) were measured in the liver 72 hours post treatment. Modification of the gap resulted in marked reduction of antisense activity and abrogation of ALT increase for both ASO sequences. Standard deviation is shown.

Supplemental Figure 6. Comparing the effect of pre-mRNA transcript length and putative site quality on transcript modulation but LNA ASOs dosed at 33 mg/kg for 24 hours. Two parameters describing the quality of putative ASO:RNA cleavage sites are represented: Since multiple putative sites can be found on a given pre-mRNA transcript, sites with the lowest number of mismatches to an ASO for each pre-mRNA transcripts and site with longest contiguous match quality are represented. The number of putative site (7 to 16 bases long) per pre-mRNA transcripts are also shown. Those 3 parameters are compared to either pre-mRNA length or log2 fold change. (A-G) Data from the 4 most toxic LNA ASOs dose at 33 mg/kg are combined. Transcription changes are filtered to display only significant changes FDR ≤ 0.01. (H-T) Same as A-G but plotted for each ASO assayed at 33 mg/kg separately. Transcription changes are filtered to display only significant changes FDR ≤ 0.1. The lower filter stringency was chosen to emphasize changes occurring following treatment with well tolerated ASOs which induced much lower level of transcript modulation. (U-AD) Same as H-T but no filtered were applied.

Supplemental Figure 7. The subchronic tolerability of 3 different cEt ASOs was assessed in Balb/c mice. (A) ALT levels were measured at 12 weeks in the plasma of Balb/c mice treated with 3 different cEt ASOs (50 mg/kg) administered weekly. (B) Off-target mRNA transcripts with long pre-mRNA (Adk, Fars2, Fbxl17, Rptor) were measured by RT-PCR in the liver of mice treated for 12 weeks with the cEt ASOs.

Supplemental Figure 8. Reanalysis of data from Kakiuchi-Kiyota et al (http://www.dtd.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53230). The authors used affymetrix Array 430 2.0. List of Illumina probes strongly correlated with ALT increase were cross-referenced with those on the affymetrix array and used for hierarchical clustering. (A) Oneway hierarchical clustering of transcripts significantly modulated in the liver at 24 hours post treatment by LNA ASOs in correlation with ALT increase at 96 hours. (Prob>0.000001, r2>0.54) shown in figure 3A are applied to the transcription profiles generated by the authors. (B) Comparison of the magnitude of transcript knockdown as a function of pre-mRNA length following treatment with the LNA ASO HTS-3 for 16 hours. HTS-3 is the most hepatotoxic ASO described by the authors.

Supplemental method

Pathway analysis: A total of 1788 transcripts that had a likely (Benjamini-Hochberg corrected $q<0.01$) fold change greater than 2 were used for the pathway analysis. Illumina transcript identifiers were imported into Ingenuity Pathway Analysis (IPA) software. The "Core Analysis" function included in IPA was used to interpret the mouse data in the context of canonical pathways. Both up- and down-regulated identifiers were defined as value parameters for the analysis. Significance of the canonical pathways was tested by the Fisher Exact test p-value.

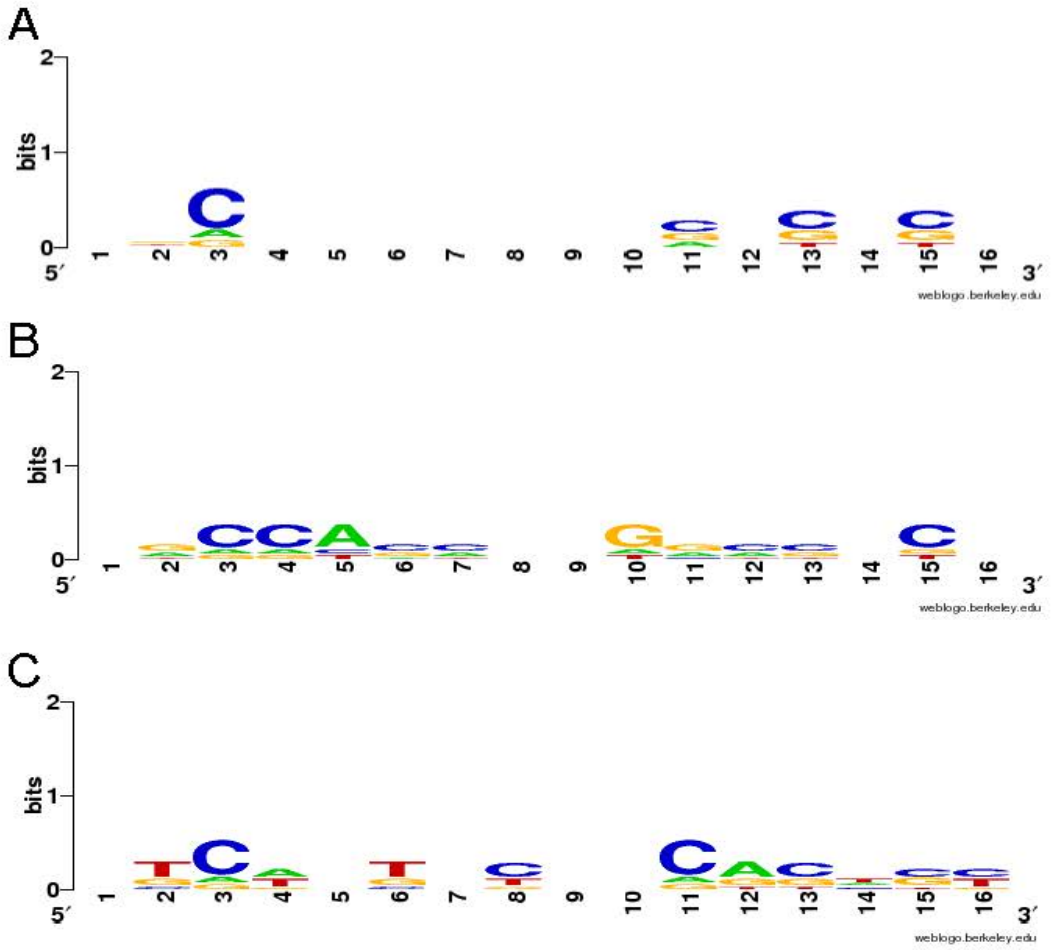
Supplemental Table 1

mRNA Target Name	Genbank number	Forward Primer	Reverse Primer	Probe
RNase H1	AF048993.1	5'-ACTCAGGATTTGTGGGCAATG-3'	5'-CCTCAGACTGCTTCGCTCCTT-3'	5' FAM-AGAGGCCGACAGACTGGCACGG-TAMRA 3'
RPTOR	NM_028898.2	5'-GCCCTCAGAAAGCTCTGGAA-3'	5'-TAGGGTCGAGGCTCTGCTTGT-3'	5' FAM-CCATCGGTGCAAACCTACAGAAGCAGTATG-TAMRA 3'
FTO	NM_011936.2	5'-AGCAGCCTACAACGTGACTTTG-3'	5'-CCACCAGGTTCTCATCGTGAT-3'	5' FAM-TTCATGGATCCTCAGAAGATGCCCTACTTG-TAMRA 3'
PPP3CA	NM_008913.1	5'-GCCCTCTGACGCCAACCT-3'	5'-CTGTCCGTGCCGTTAGTCTCT-3'	5' FAM-AACTCCATCAACAAGGCTCTCGCC-TAMRA 3'
PTPRK	NM_008983.2	5'-GAAACTAAAACCCAATGTGTACGAATT-3'	5'-GATGCCCGCGATTTTCAC-3'	5' FAM-AAGAACCAGAAGTGATCCCAGACCCGG-TAMRA 3'
IQGAP2	NM_027711.1	5'-GACCTACCAGCAACTGTTCTAC-3'	5'-GAGTGAAGATAACCGTGTCCAT-3'	5' FAM-AGACCAAACCTTCATACCTGGCTAAGC-TAMRA 3'
CHN2	NM_023543.2	5'-CACAACGACAACCACTTCAAC-3'	5'-GGCACAGTATTCACACCAGT-3'	5' FAM-CAACTTCAAGGTGCACACATTCCGG-TAMRA 3'
FBXL17	NM_015794.1	5'-CGGATGGTGTAAAGAAATCACAGA-3'	5'-ATCTCATCAGGCCCAAGTATCTCA-3'	5' FAM-CAAGGAGCCACCCTGATTGCACAGA-TAMRA 3'
F7	NM_010172.3	5'-AATGAGGAACAGTGCTCCTTTGA-3'	5'-TGTAAACAATCCAGAACTGCTTGGT-3'	5' FAM-CCCGGGAGATCTTCAAGAGCCC-TAMRA 3'

Supplemental Table 2

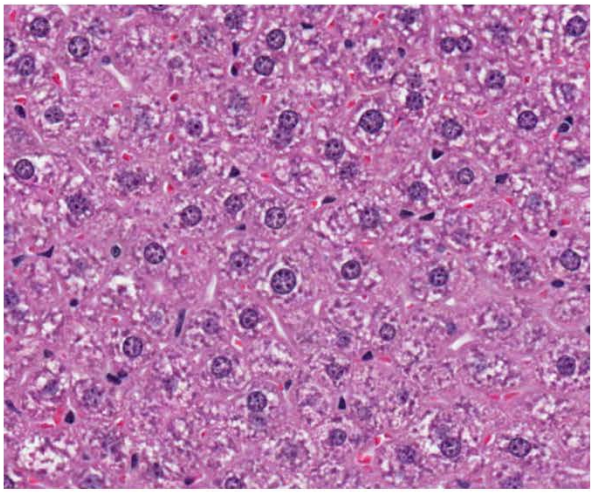
rank	1st Toxic Dose (mg/kg) >1000 IU/ml ALT (96h)	Alternative ID	ISIS No	Target Gene	Sequence	0	1	2	3	4	5	6	7	8	9
1	>300	FVII-1	571035	*F7*	5'-CAGATATAGGACTGGA-3'	0	16	189	1158	3203	3568	2254	954	355	89
2	>300	Gen2.5 ctrl	569713	-	5'-GACGCGCCTGAAGGTT-3'	0	0	25	355	2028	4371	3340	1246	434	78
3	>300	FXI-4	569714	*F11*	5'-GGCCACCACGCTGTCA-3'	2	11	124	982	3099	3989	2614	864	309	65
4	>300	FXI-5	571034	*F11*	5'-TGCCACCGTAGACACG-3'	1	1	33	430	2074	3929	3136	1357	481	117
5	>300	FXI-3	571033	*F11*	5'-ATCCAGAGATGCCTCC-3'	1	9	331	1688	4133	3859	1372	609	233	68
6	>300	SOD1-1	569715	*Sod1*	5'-GGACACATTGGCCACA-3'	2	10	218	1385	3729	3524	2094	888	280	76
7	300	FVII-2	569716	*F7*	5'-CCCTGGTGTACACCCC-3'	1	6	109	925	3156	3823	2453	1026	375	128
8	300	BIRC5	554219	*Birc5*	5'- CTCAATCCATGGCAGC-3'	0	13	143	1274	3341	3908	2168	893	319	90
9	100	FXI-2	569720	*F11*	5'-GTCAGTATCCCAGTGT-3'	1	2	148	1183	3229	3803	2295	948	309	109
11	100	FVII-3	569718	*F7*	5'-TGGTCCCTGCAGTACT-3'	1	8	192	1228	3457	3973	2226	765	238	62
10	33	PTEN	569717	*Pten*	5'-ATCATGGCTGCAGCTT-3'	1	11	264	1523	3754	3769	2011	762	235	47
12	33	SOD1-2	569721	*Sod1*	5'-TGAGGTCCTGCACTGG-3'	1	14	224	1377	3381	3958	2177	731	249	38
13	11	FXI-1	569719	*F11*	5'-GTCTGTGCATCTCTCC-3'	1	18	337	2003	3853	3387	1591	636	254	58

Supplemental Figure 1

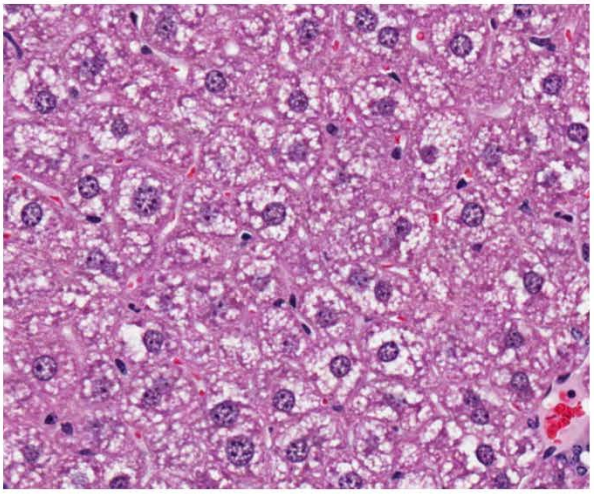


Supplemental Figure 2

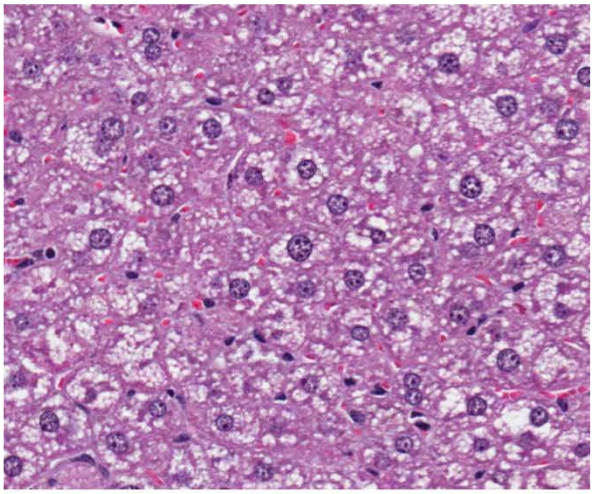
Saline
Normal liver morphology



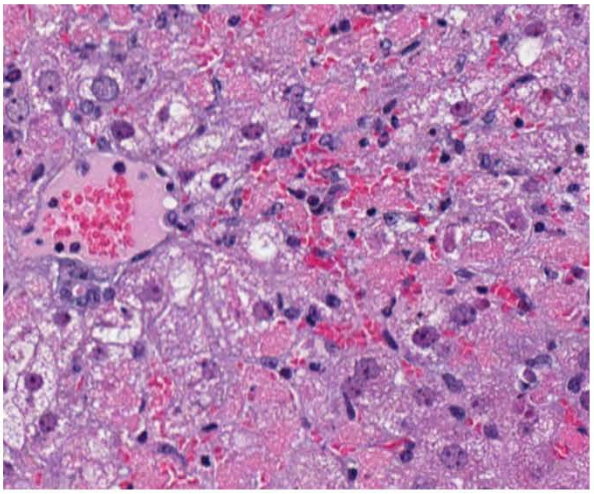
ISIS 569720 (FXI-2) 11 mg/kg – 96h
Mild to moderate microvasicular changes



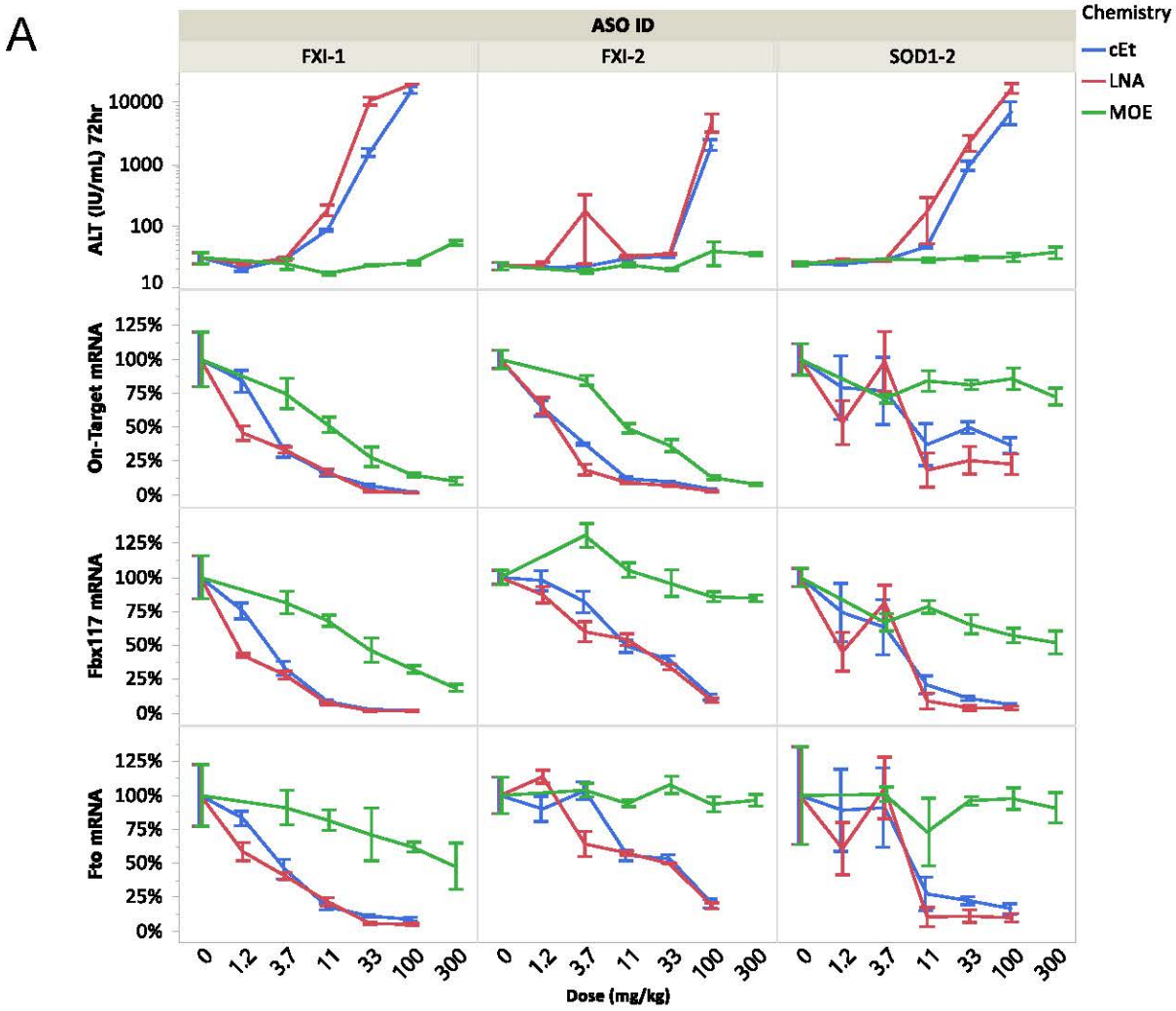
ISIS 569720 (FXI-2) 33 mg/kg – 96h
Mild to moderate microvasicular changes

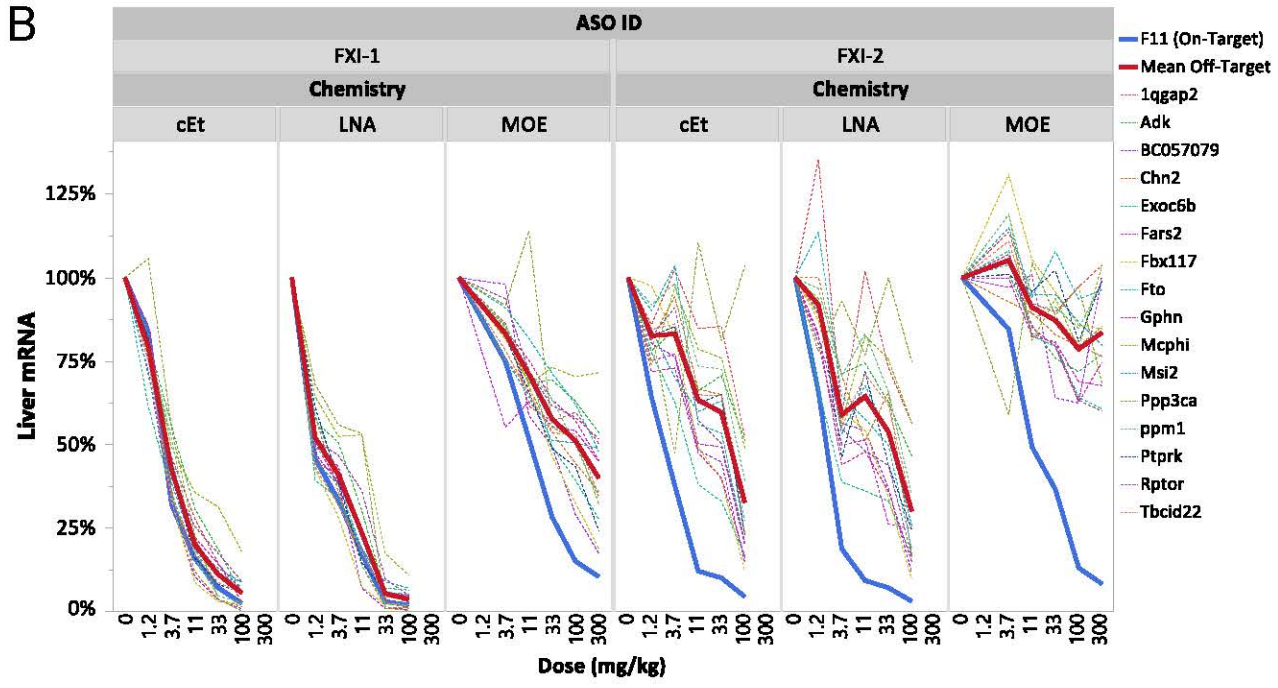


ISIS 569720 (FXI-2) 100 mg/kg – 96h
Severe zonal (zone 1) necrosis



Supplemental Figure 3

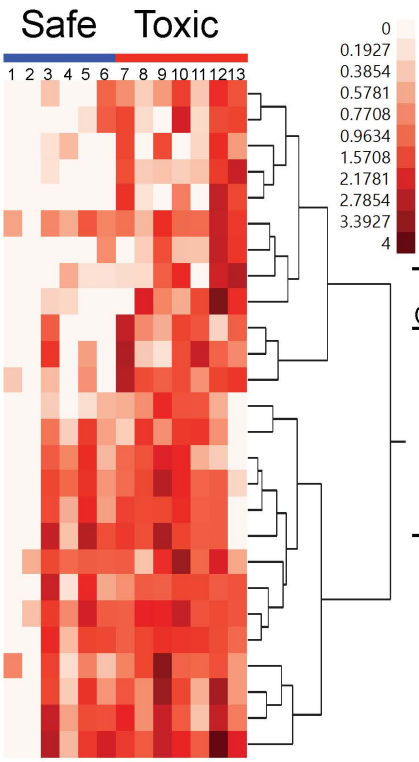




Supplemental Figure 4

A

- Hereditary Breast Cancer Signaling
- Role of BRCA1 in DNA Damage Response
- JAK/Stat Signaling
- TR/RXR Activation
- Cell Cycle Control of Chromosomal Replication
- Breast Cancer Regulation by Stathmin1
- ATM Signaling
- Role of CHK Proteins in Cell Cycle Checkpoint Control
- Molecular Mechanisms of Cancer
- Glycosphingolipid Biosynthesis - Lactoseries
- O-Glycan Biosynthesis
- Rac Signaling
- Relaxin Signaling
- Androgen Signaling
- Dopamine-DARPP32 Feedback in cAMP Signaling
- Synaptic Long Term Depression
- Melatonin Signaling
- Neuropathic Pain Signaling In Dorsal Horn Neurons
- Role of Tissue Factor in Cancer
- Thrombin Signaling
- CXCR4 Signaling
- α-Adrenergic Signaling
- Endothelin-1 Signaling
- Renin-Angiotensin Signaling
- EGF Signaling
- Non-Small Cell Lung Cancer Signaling



Column	Toxicity	ASO ID	Dose (mg/kg)
1	Safe	Gen2.5 ctrl	300
2		FVII-1	300
3		FXI-3	300
4		FXI-4	300
5		FXI-5	300
6		SOD1-1	300
7	Severe	BIRC5	300
8		FVII-2	300
9		FXI-2	100
10		PTEN	100
11		FVII-3	100
12		SOD1-2	33
13		FXI-1	33

B

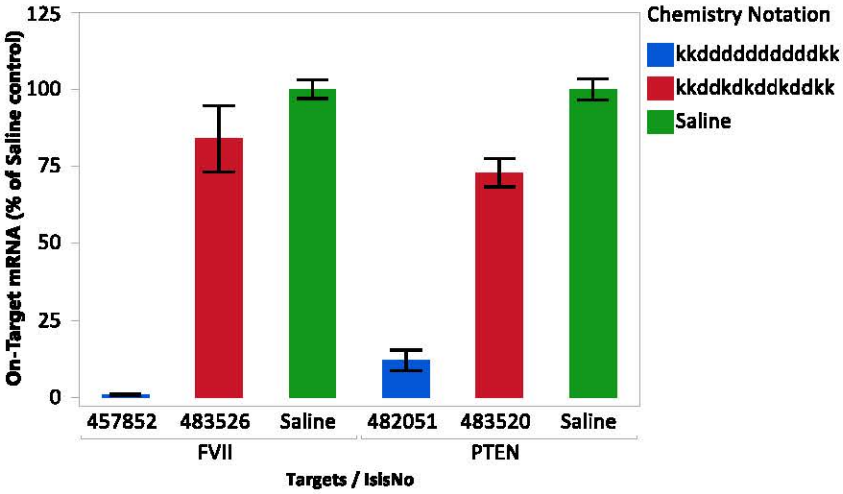
Modulation	Ingenuity Canonical Pathways	-log (p-value)	Transcripts
Top 10 Upregulated Pathways	EIF2 Signaling	8.71	RPL24, EIF2S3, EIF2B4, RPS19, RPS8, RPS21, EIF2B2, RPL7, RPS4X, RPS10, RPS26, EIF4A1, EIF5, RPL35, RPL19, RPLP2, RPL18
	Regulation of eIF4 and p70S6K Signaling	4.98	EIF2S3, RPS4X, PPP2R1A, EIF2B4, RPS10, RPS26, EIF4A1, RPS19, RPS8, RPS21, EIF2B2
	Hereditary Breast Cancer Signaling	3.55	TP53, NPM1, POLR2A, POLR2D, GADD45A, H2AFX, CDK4, POLR2H
	Pyrimidine Metabolism	3	NME3, POLR2A, POLR2D, PNP, POLR2H, NUDT2, PUS1, UMPS
	Aminoacyl-tRNA Biosynthesis	2.89	NARS, RARS, QARS, IARS
	mTOR Signaling	2.73	RPS4X, PPP2R1A, RPS10, RPS26, EIF4A1, RPS19, RPS8, RPS21, MLST8
	GADD45 Signaling	2.6	TP53, GADD45A, CDK4
	One Carbon Pool by Folate	2.53	MTHFD2, ATIC, SHMT2
	Inositol Metabolism	2.51	ALDOA, ALDOC
	Role of CHK Proteins in Cell Cycle Checkpoint Control	2.1	TP53, PPP2R1A, HUS1, ATMIN
Top 10 Downregulated Pathways	Breast Cancer Regulation by Stathmin1	4.32	ADCY9, MAP2K2, ITPR2, PPM1L, PIK3C2G, PRKAG2, ARHGEF11, ITPR1, PPP1CA, GNG12, PRKCA
	α-Adrenergic Signaling	3.83	ADCY9, MAP2K2, ITPR2, PRKAG2, ITPR1, GNG12, PRKCA
	CXCR4 Signaling	3.74	ADCY9, DOCK1, MAP2K2, ITPR2, PIK3C2G, ARHGEF11, ITPR1, GNG12, PRKCA
	Gap Junction Signaling	3.55	ADCY9, MAP2K2, ITPR2, PIK3C2G, PRKAG2, ITPR1, MAP2K5, PPP3CA, PRKCA
	Synaptic Long Term Potentiation	3.35	MAP2K2, ITPR2, PRKAG2, ITPR1, PPP1CA, PPP3CA, PRKCA
	fMLP Signaling in Neutrophils	3.25	MAP2K2, ITPR2, PIK3C2G, ITPR1, GNG12, PPP3CA, PRKCA
	Renin-Angiotensin Signaling	3.23	ADCY9, MAP2K2, ITPR2, PIK3C2G, PRKAG2, ITPR1, PRKCA
	Role of NFAT in Cardiac Hypertrophy	3.14	ADCY9, MAP2K2, ITPR2, PIK3C2G, PRKAG2, ITPR1, GNG12, PPP3CA, PRKCA
	CCR3 Signaling in Eosinophils	3.11	MPRIP, MAP2K2, ITPR2, PIK3C2G, ITPR1, GNG12, PRKCA
	Protein Kinase A Signaling	3.05	ADCY9, MAP2K2, ITPR2, SMAD3, RYR3, PRKAG2, ITPR1, PPP1CA, AKAP7, GNG12, PPP3CA, PRKCA

Supplemental Figure 5

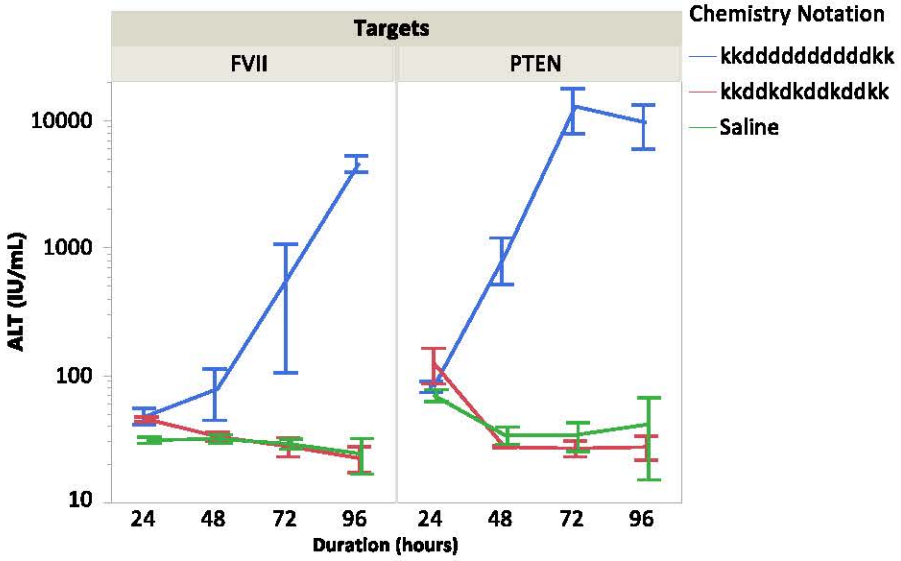
A

ASO ID	Targets	Dose (mg/kg)	Sequence (red=cEt)	Chemistry Notation (k=cEt, d=deoxy)
482051	PTEN	200	TCATGGCTGCAGCT	kkddddddddddkk
483520			TCATGGCTGCAGCT	kkddkdkddkddkk
457852	FVII	50	GGTCCCTGCAGTAC	kkddddddddddkk
483526			GGTCCCTGCAGTAC	kkddkdkddkddkk

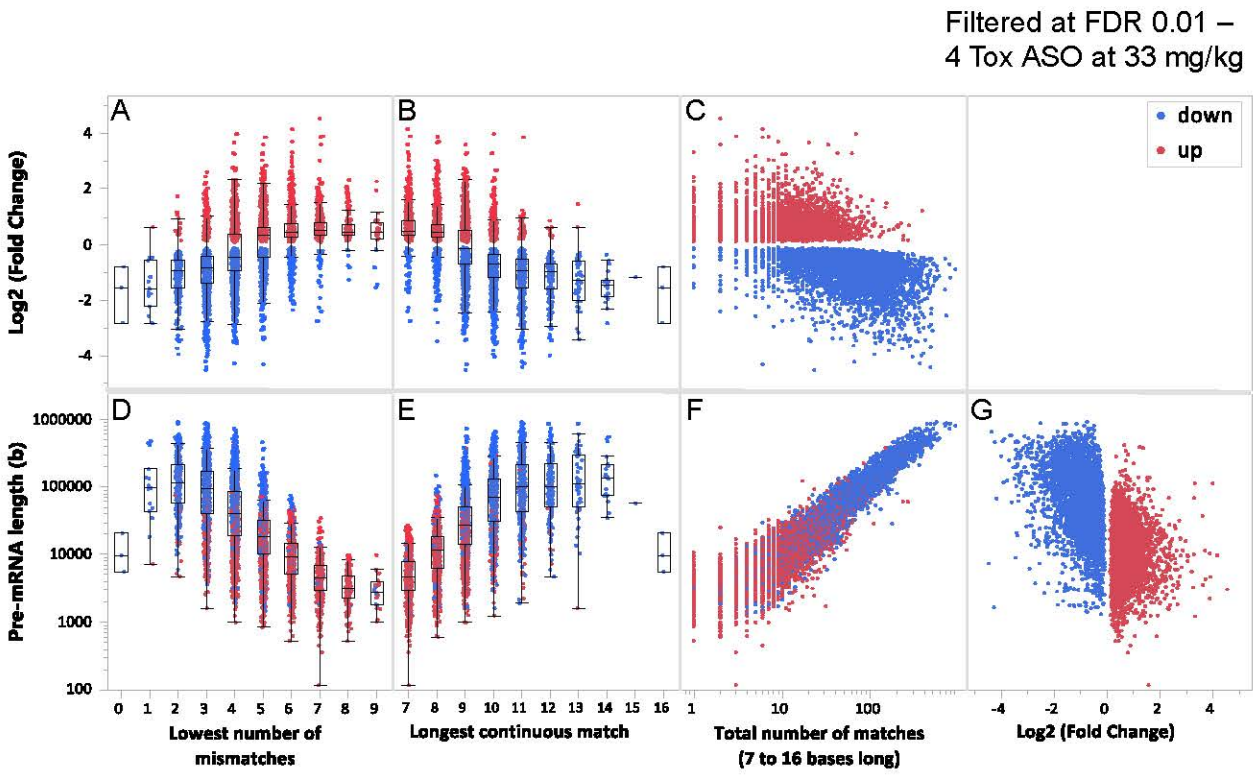
B



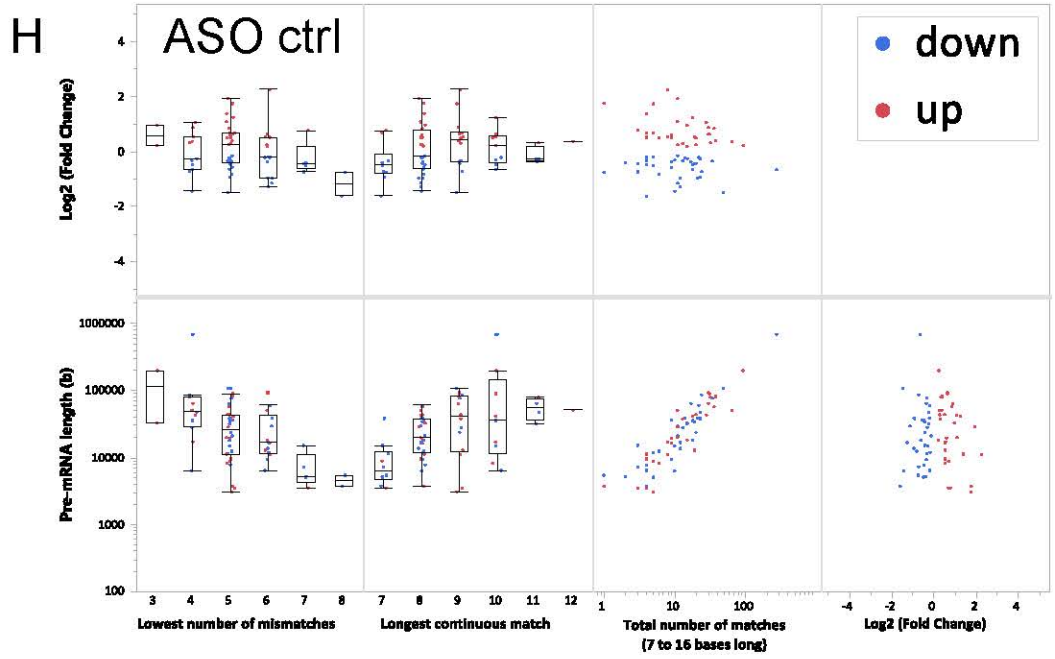
C

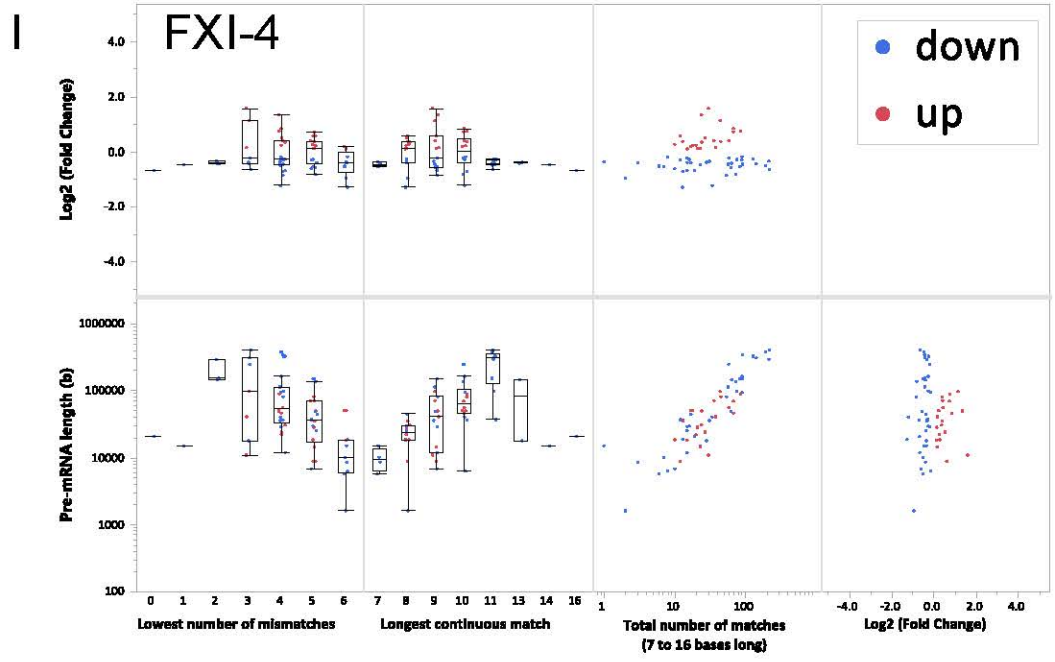


Supplemental Figure 6



Supplemental Figure 6

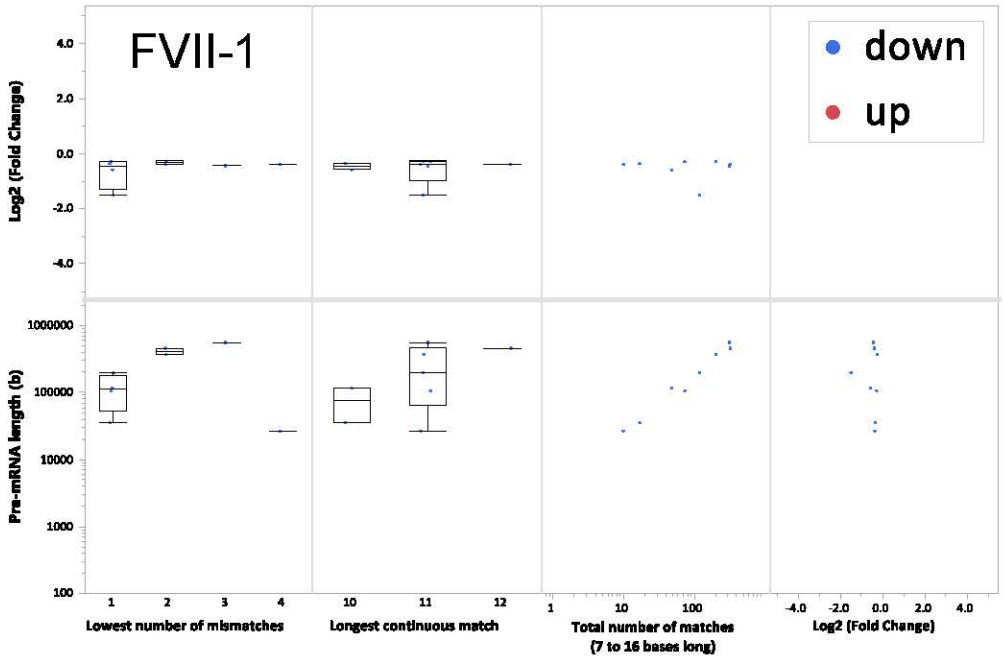




	ASO ID	Alt. ID	Dose (mg/kg)	Toxicity (96 h)	ALT (IU/mL) 96 hours
Top graph	569713	ASO ctrl	**33**	**Safe**	**23**
			300	Safe	27
Bottom graph	569714	FXI-4	**33**	**Safe**	**32**
			300	Safe	30

Supplemental Figure 6

J



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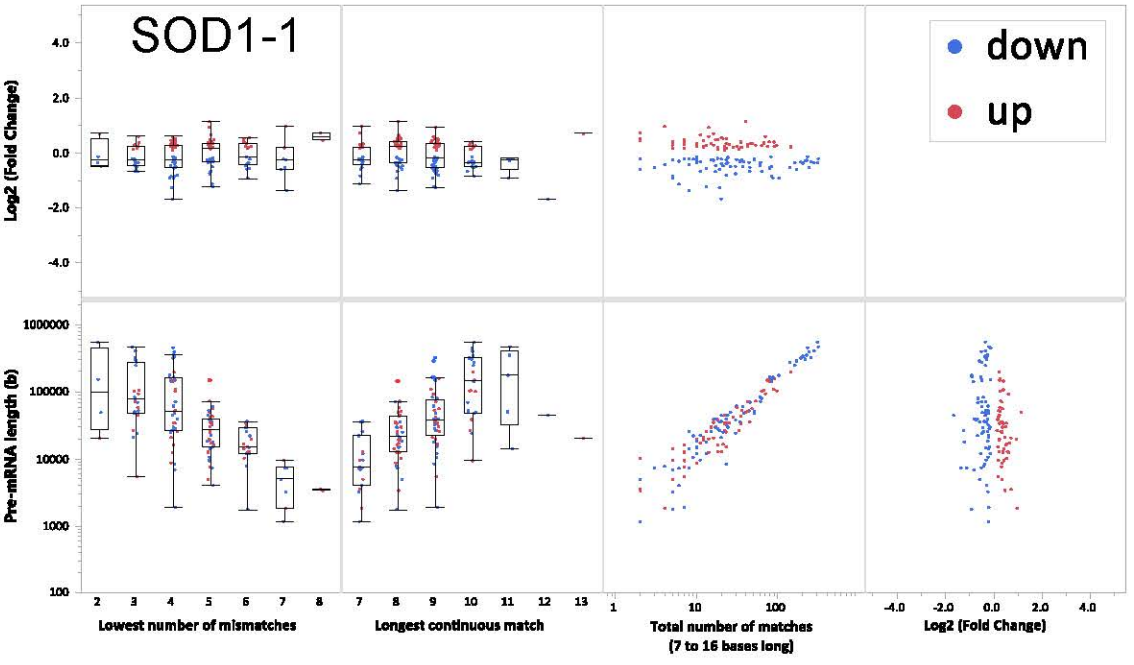
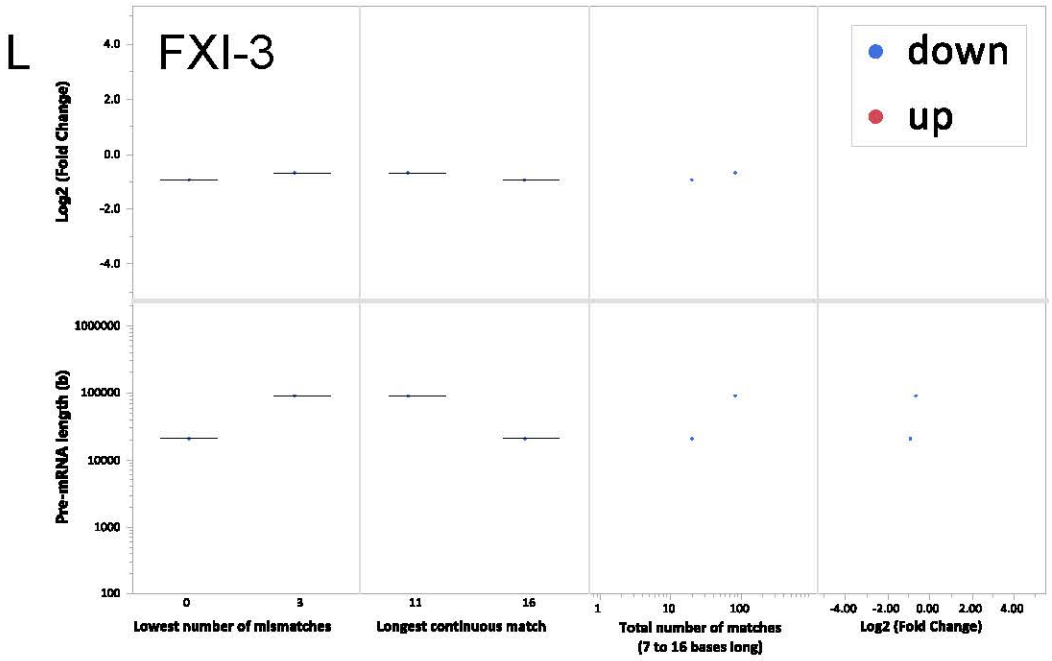
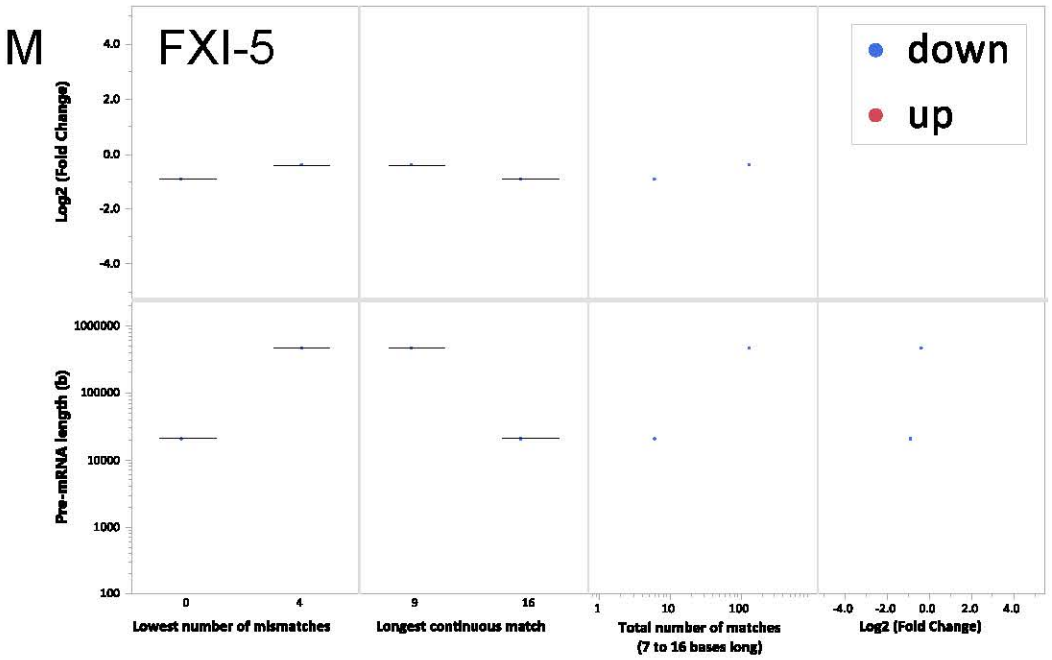


	ASO ID	Alt. ID	Dose (mg/kg)	Toxicity (96 h)	ALT (IU/mL) 96 hours
Top graph	571035	FVII-1	**33**	**Safe**	**19**
			300	Safe	93
Bottom graph	569715	SOD1-1	**33**	**Safe**	**39**
			300	Safe	87

Supplemental Figure 6





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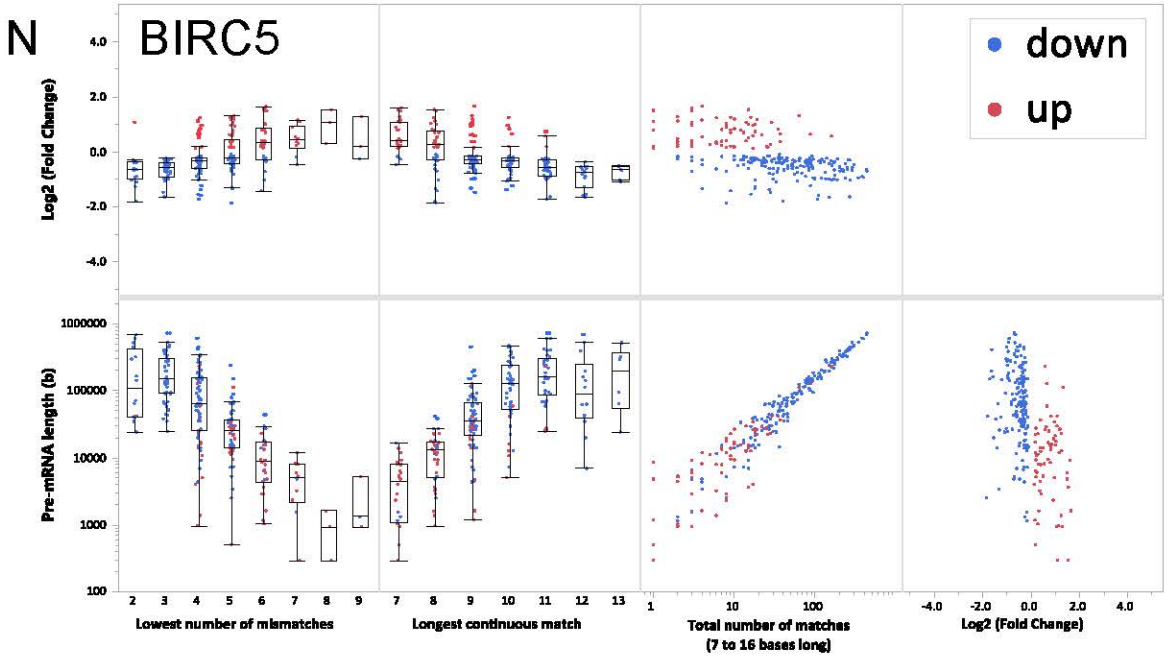
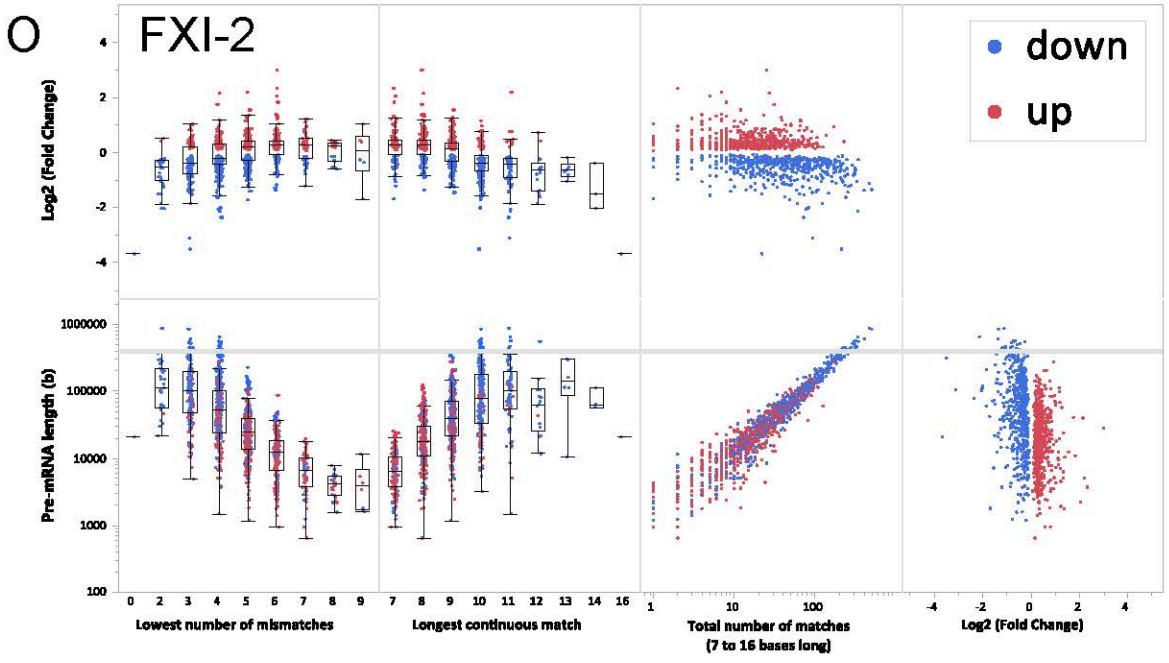
Top graph	571033	FXI-3	**33**	**Safe**	**33**
			300	Safe	121
Bottom graph	571034	FXI-5	**33**	**Safe**	**56**
			300	Safe	193

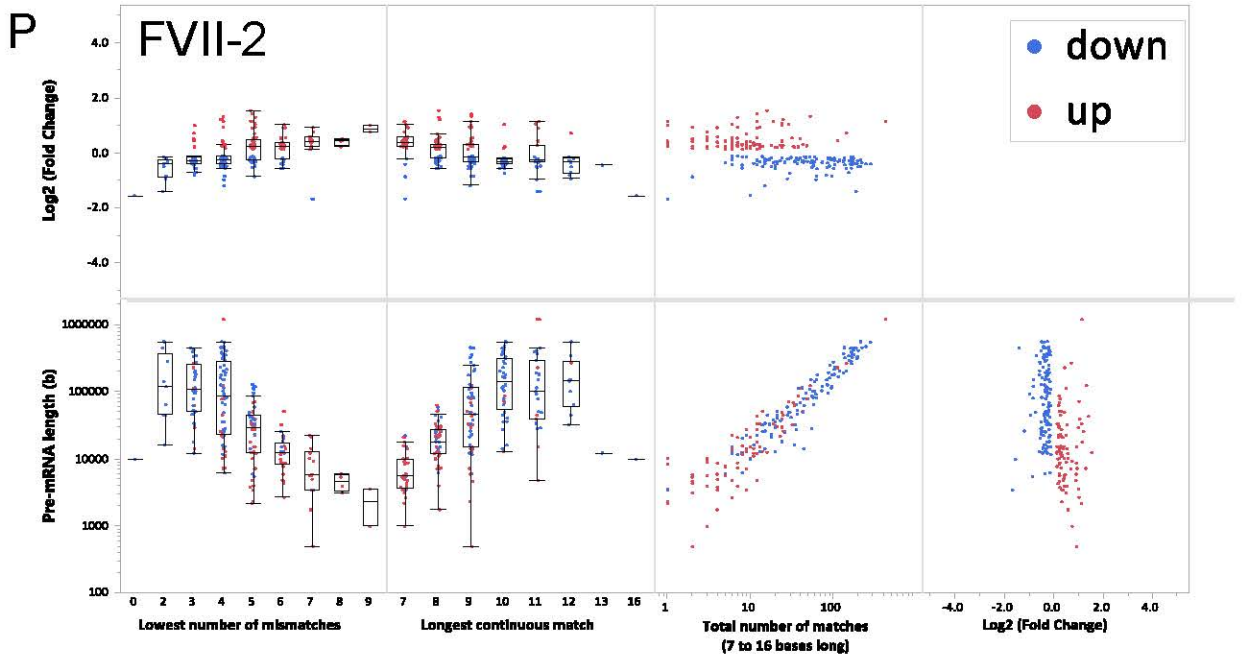
Supplemental Figure 6

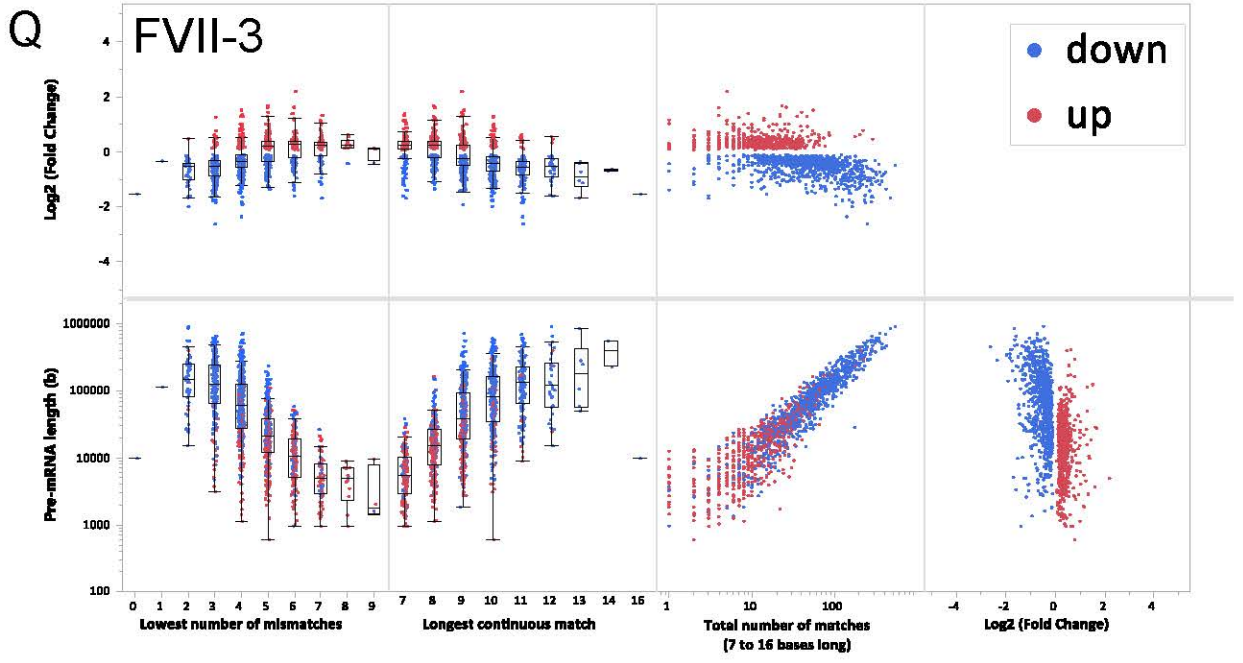




	ASO ID	Alt. ID	Dose (mg/kg)	Toxicity (96 h)	ALT (IU/mL) 96 hours
Top graph	554219	BIRC5	**33** 300	**Safe** Severe	**33** 1475
Bottom graph	569720	FXI-2	**33** 100	**Safe** Severe	**125** 10179

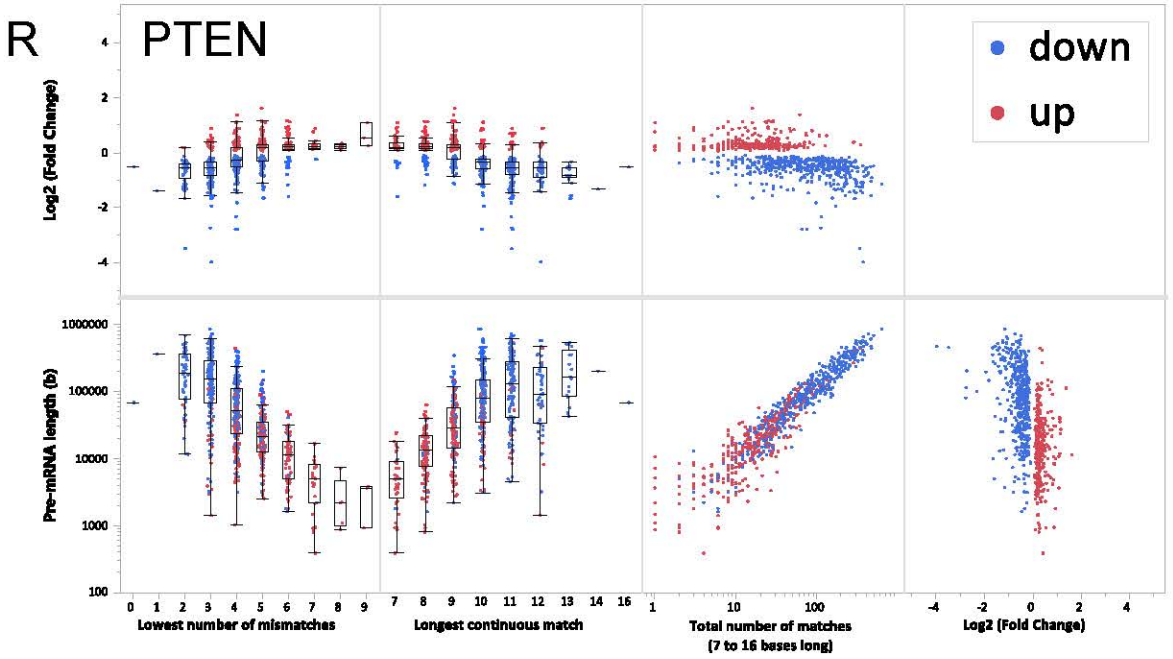
Supplemental Figure 6

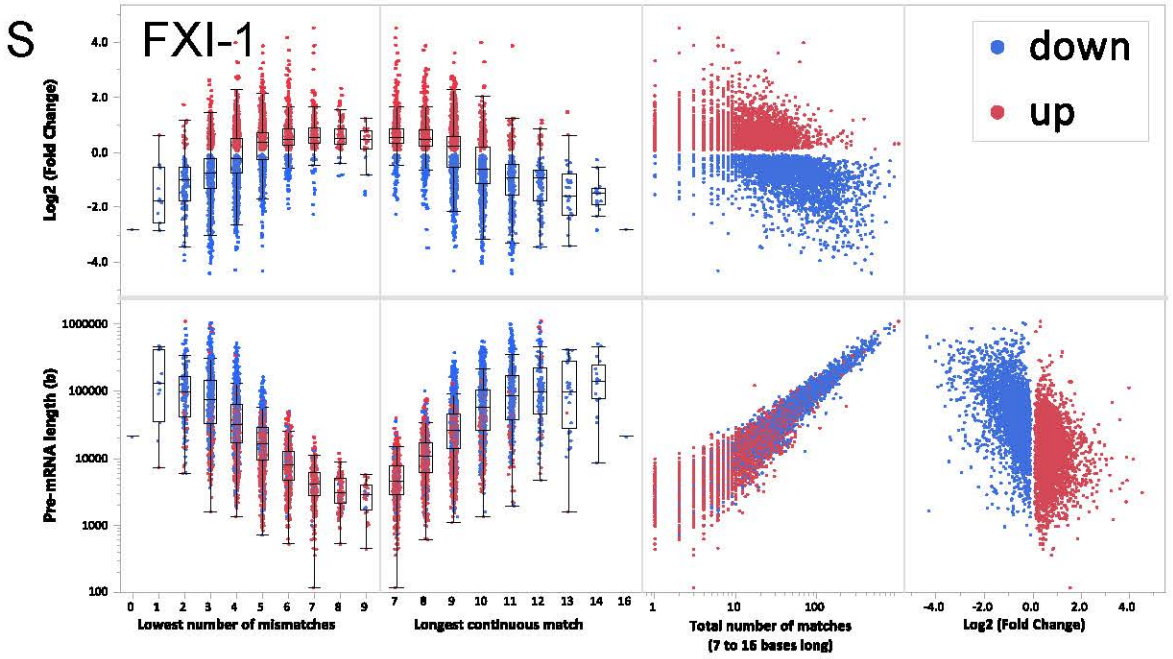




	ASO ID	Alt. ID	Dose (mg/kg)	Toxicity (96 h)	ALT (IU/mL) 96 hours
Top graph	569716	FVII-2	**33** 300	**Safe** Severe	**128** 4690
Bottom graph	569718	FVII-3	**33** 100	**Moderate** Severe	**461** 5034

Supplemental Figure 6





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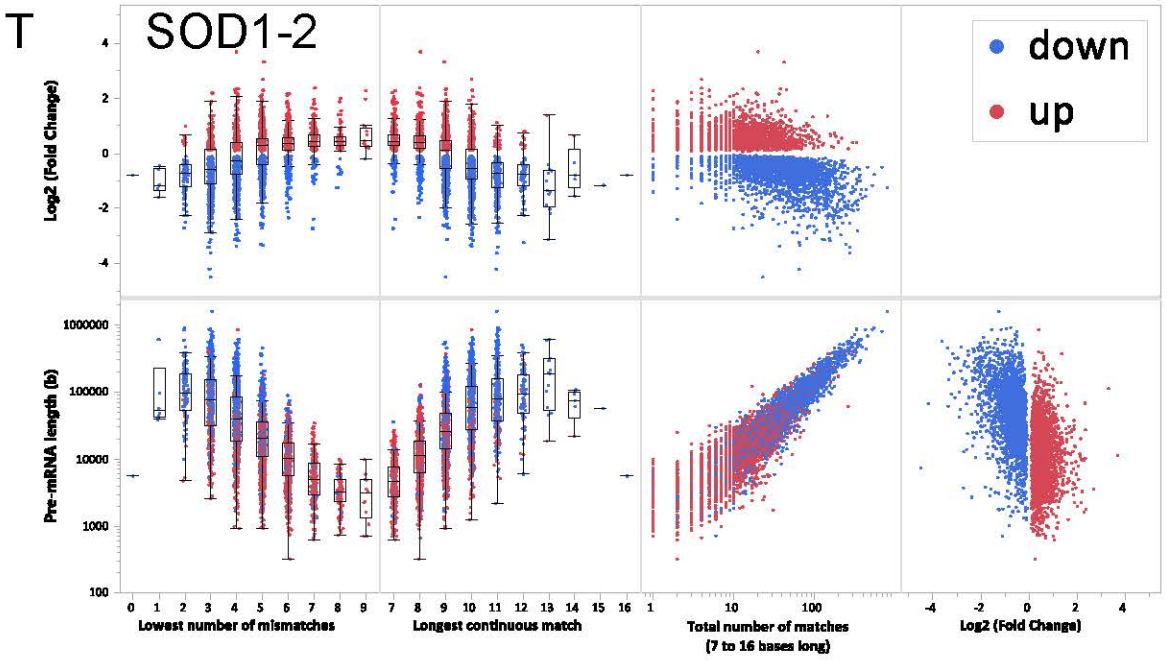
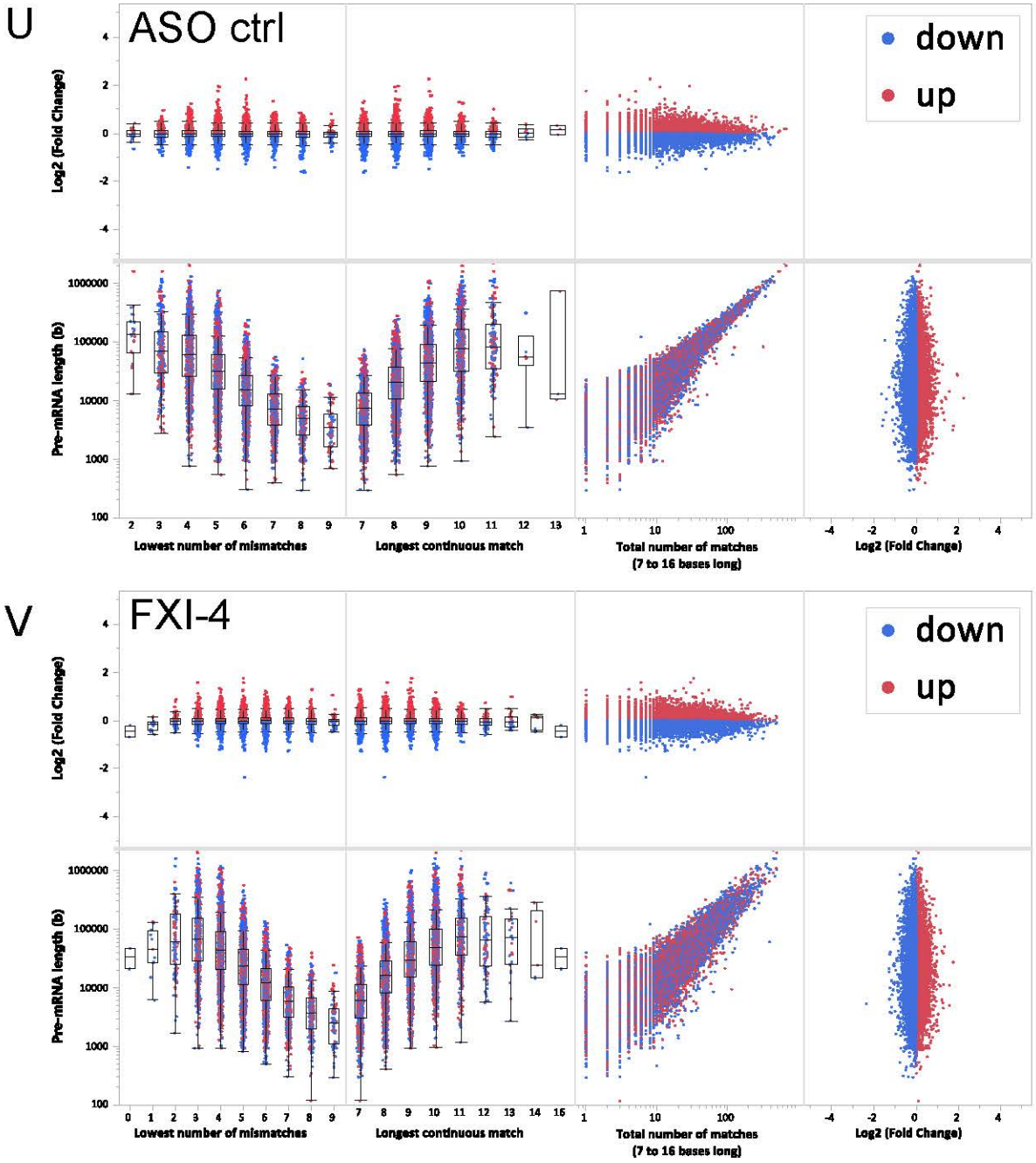
Top graph	569717	PTEN	**33**	**Severe**	**1143**
			100	Severe	10262
Bottom graph	569719	FXI-1	11	Severe	1486
			33	**Severe**	**20000**

Supplemental Figure 6



ASO ID	Alt. ID	Dose (mg/kg)	Toxicity (96 h)	ALT (IU/mL) 96 hours
569721	SOD1-2	33	Severe	2288

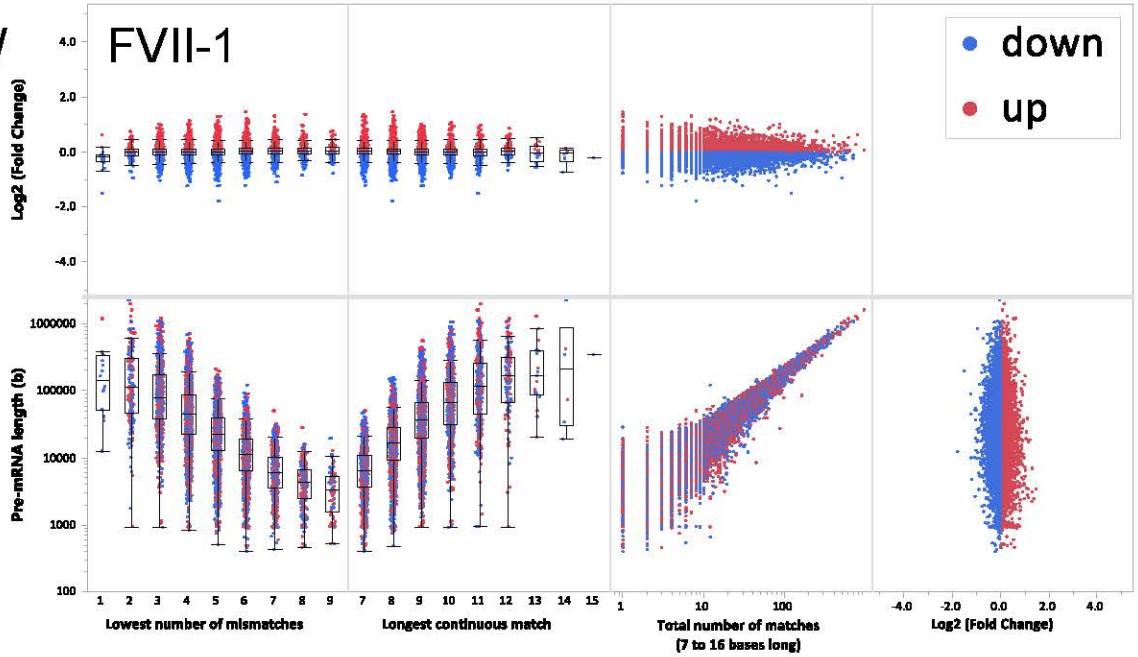
Supplemental Figure 6



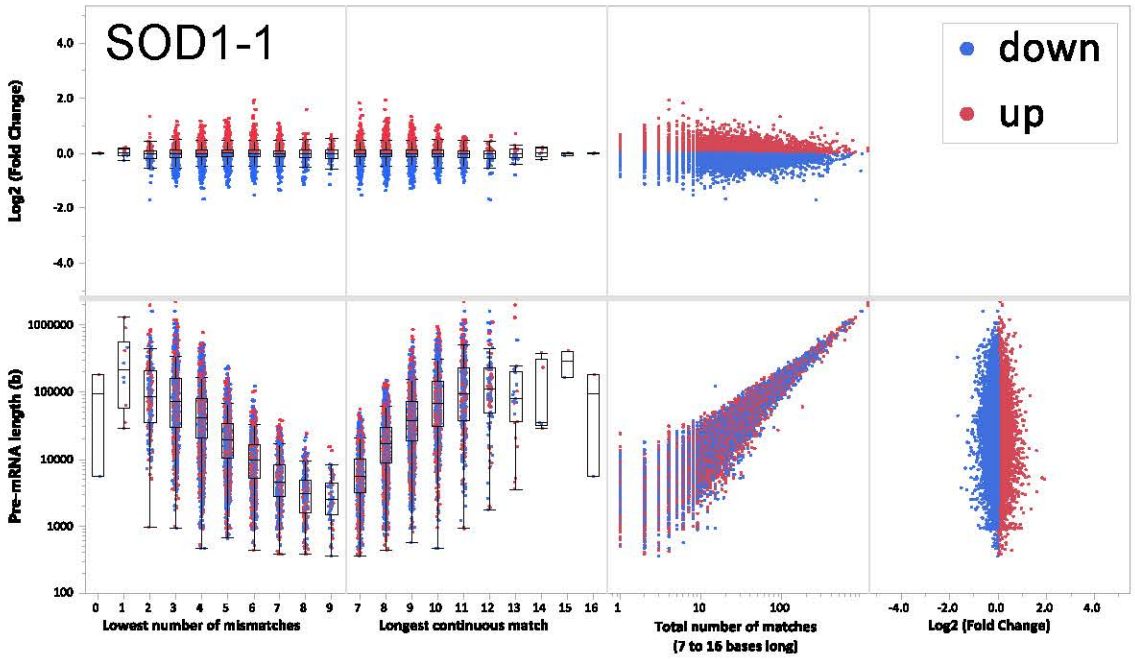
	ASO ID	Alt. ID	Dose (mg/kg)	Toxicity (96 h)	ALT (IU/mL) 96 hours
Top graph	569713	ASO ctrl	**33**	**Safe**	**23**
			300	Safe	27
Bottom graph	569714	FXI-4	**33**	**Safe**	**32**
			300	Safe	30

Supplemental Figure 6

W

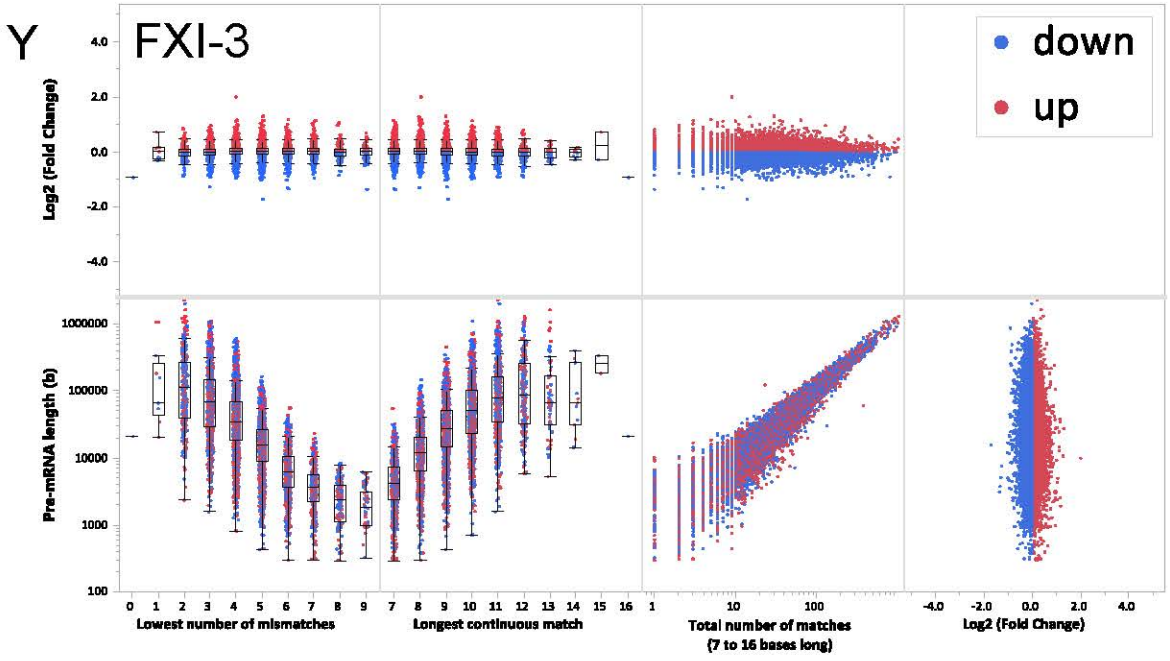


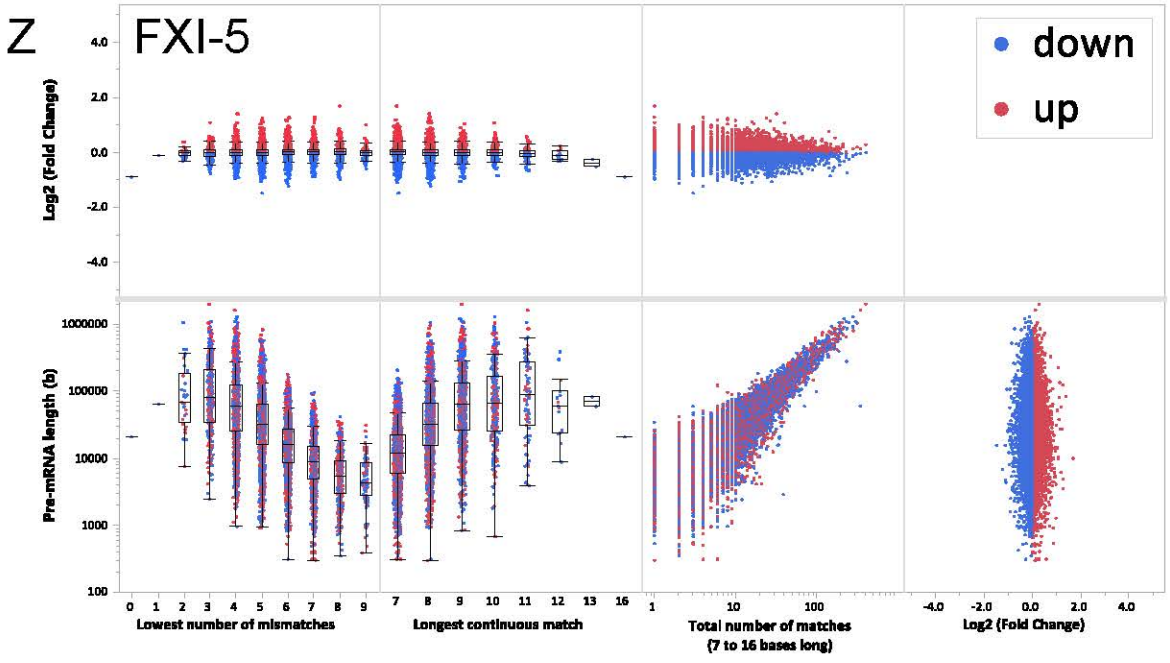
X



	ASO ID	Alt. ID	Dose (mg/kg)	Toxicity (96 h)	ALT (IU/mL) 96 hours
Top graph	571035	FVII-1	**33**	**Safe**	**19**
			300	Safe	93
Bottom graph	569715	SOD1-1	**33**	**Safe**	**39**
			300	Safe	87

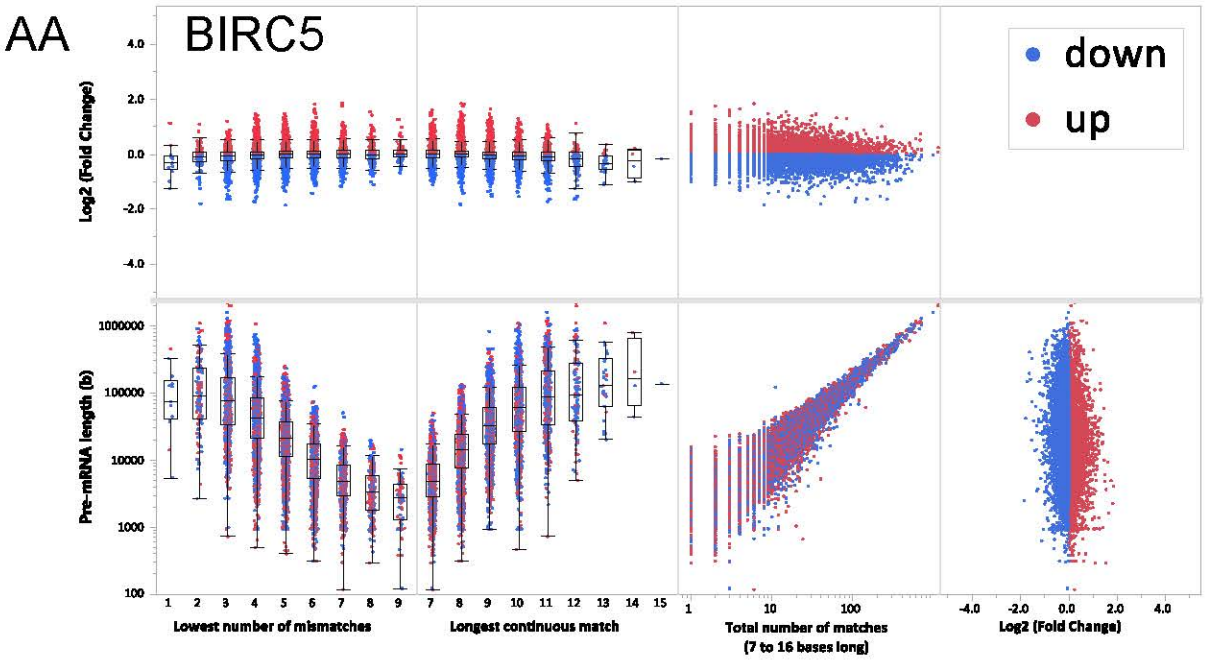
Supplemental Figure 6

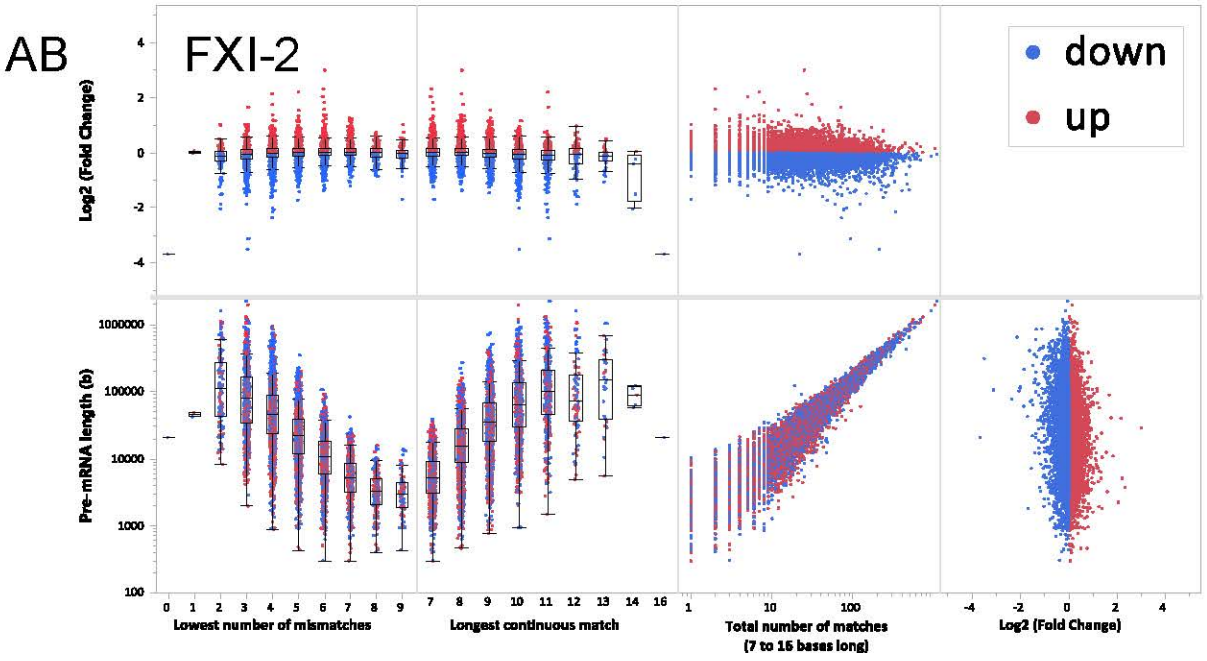




	ASO ID	Alt. ID	Dose (mg/kg)	Toxicity (96 h)	ALT (IU/mL) 96 hours
Top graph	571033	FXI-3	**33**	**Safe**	**33**
			300	Safe	121
Bottom graph	571034	FXI-5	**33**	**Safe**	**56**
			300	Safe	193

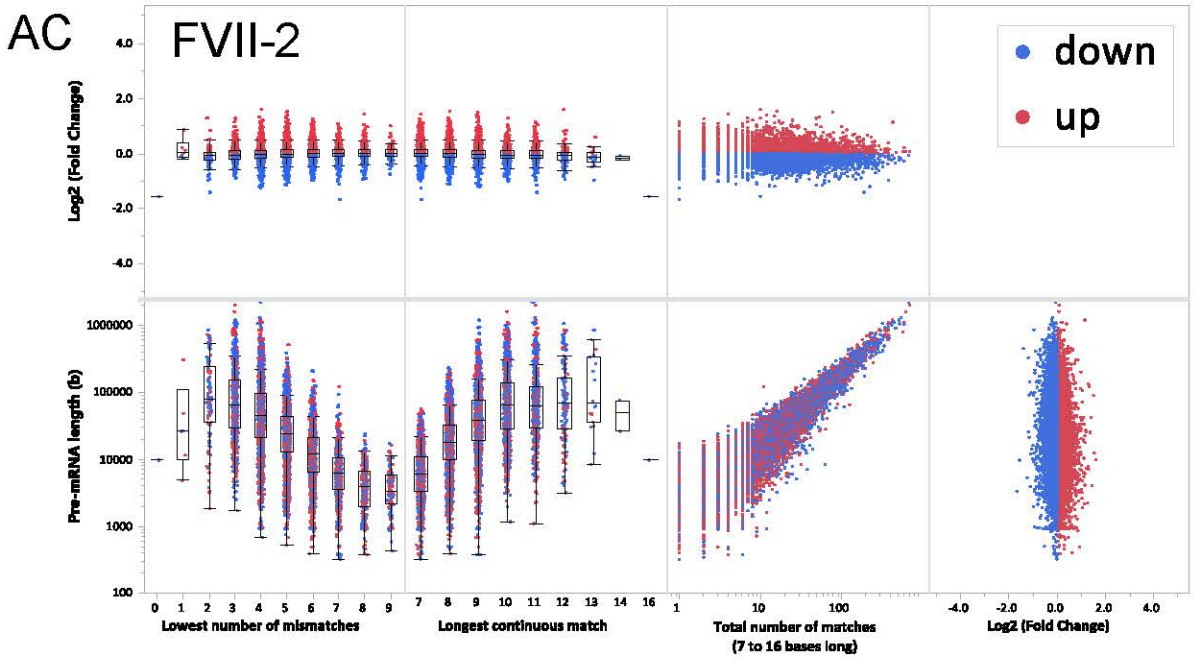
Supplemental Figure 6

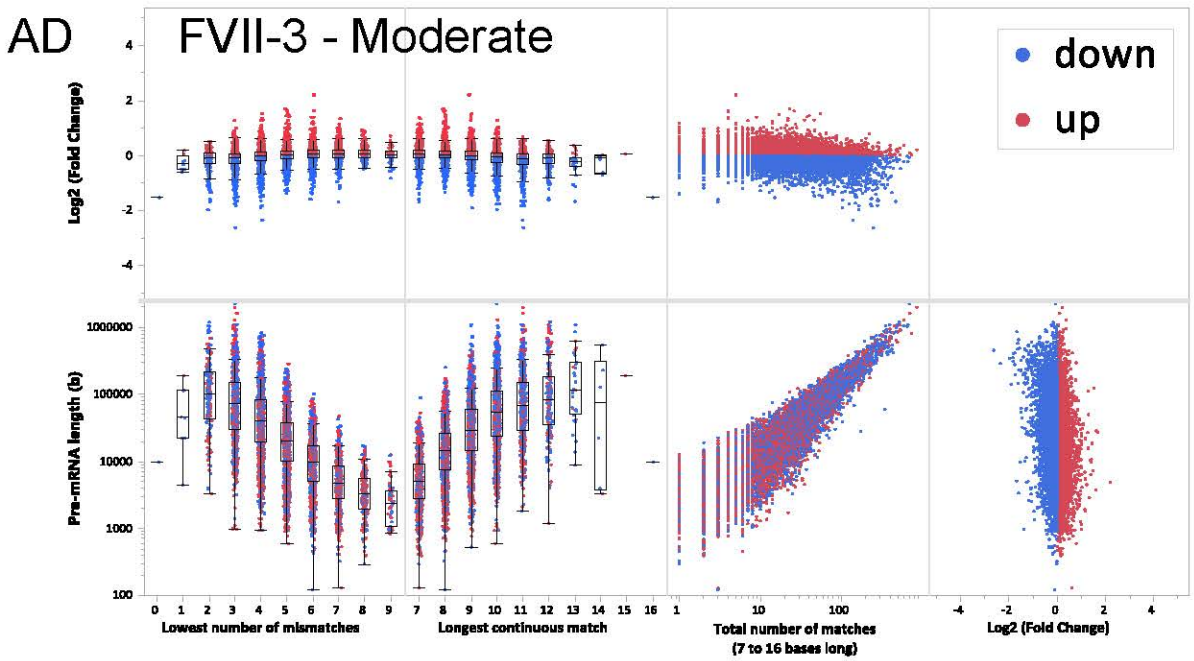




	ASO ID	Alt. ID	Dose (mg/kg)	Toxicity (96 h)	ALT (IU/mL) 96 hours
Top graph	554219	BIRC5	**33** 300	**Safe** Severe	**33** 1475
Bottom graph	569720	FXI-2	**33** 100	**Safe** Severe	**125** 10179

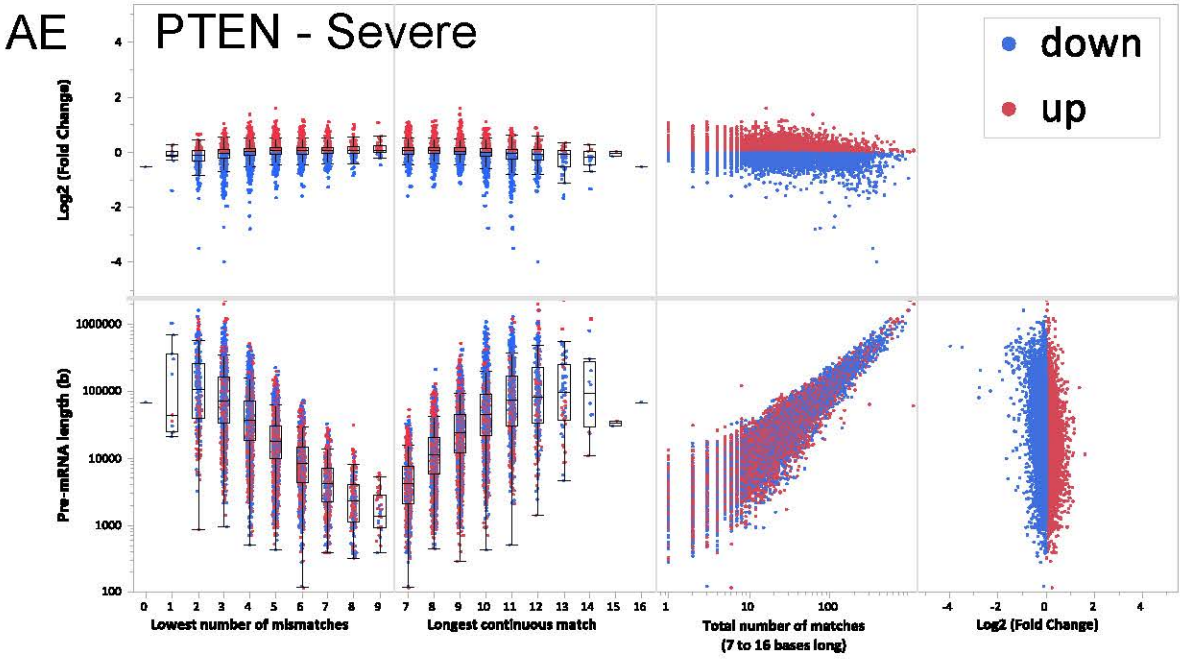
Supplemental Figure 6

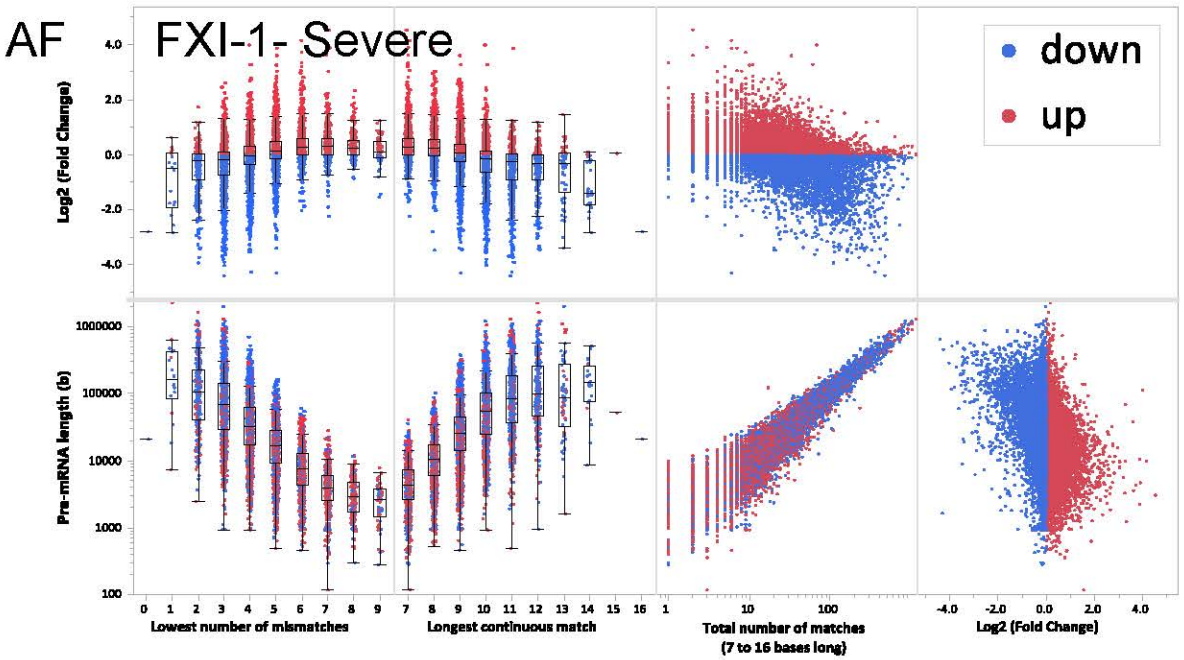




	ASO ID	Alt. ID	Dose (mg/kg)	Toxicity (96 h)	ALT (IU/mL) 96 hours
Top graph	569716	FVII-2	**33**	**Safe**	**128**
			300	Severe	4690
Bottom graph	569718	FVII-3	**33**	**Moderate**	**461**
			100	Severe	5034

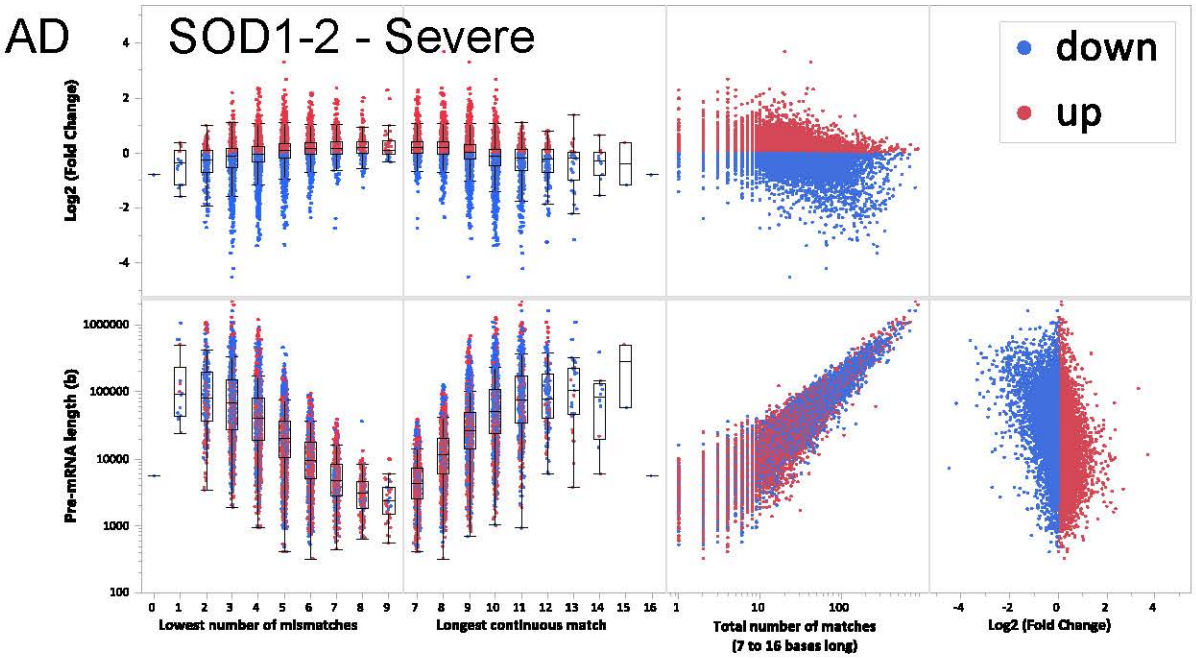
Supplemental Figure 6





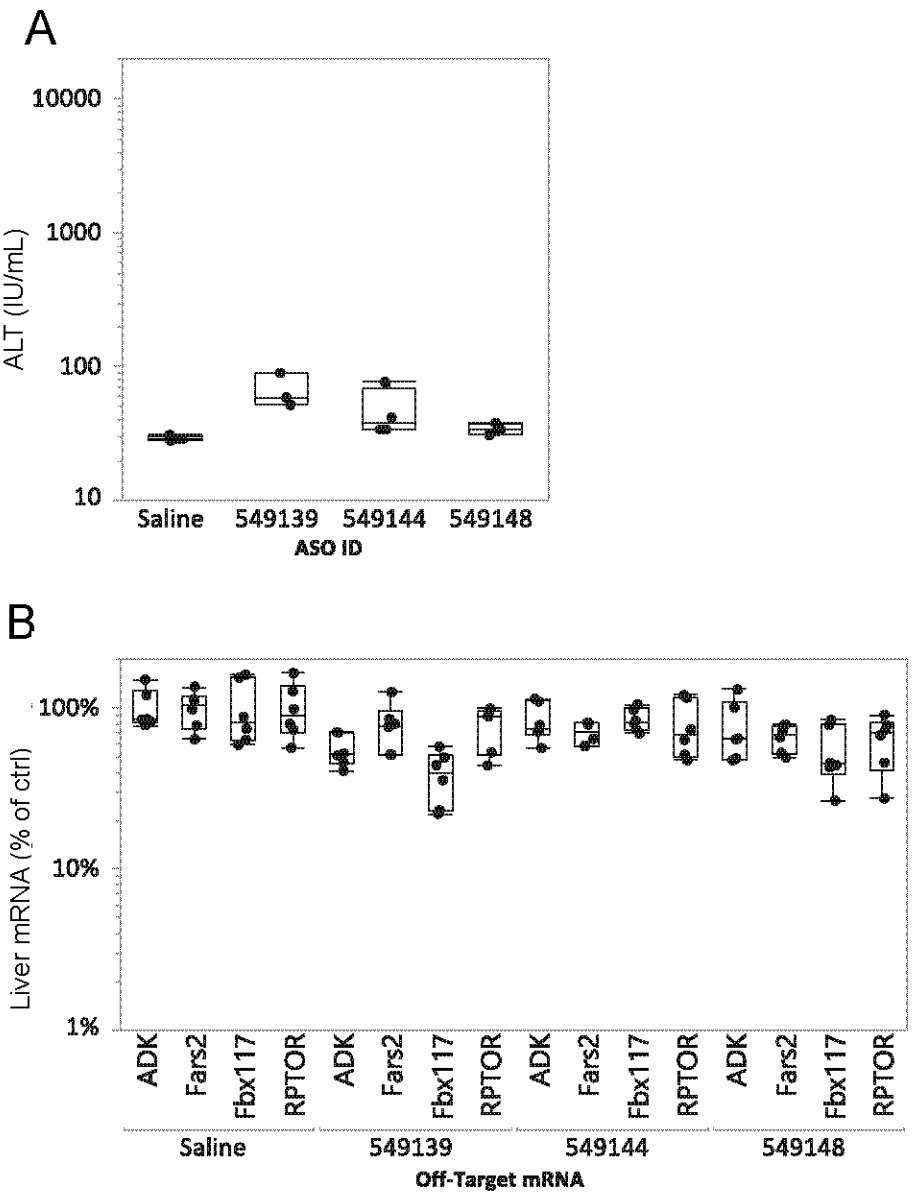
	ASO ID	Alt. ID	Dose (mg/kg)	Toxicity (96 h)	ALT (IU/mL) 96 hours
Top graph	569717	PTEN	**33**	**Severe**	**1143**
			100	Severe	10262
Bottom graph	569719	FXI-1	11	Severe	1486
			33	**Severe**	**20000**

Supplemental Figure 6



ASO ID	Alt. ID	Dose (mg/kg)	Toxicity (96 h)	ALT (IU/mL) 96 hours
569721	SOD1-2	33	Severe	2288

Supplemental Figure 7



ISIS No	Sequence	modification	Gene	Genbank number	On-target Species
549139	5'-GACGCGCCTGAAGGTT-3'	cEt	N/A, control	N/A	N/A
549144	5'-GGCCAATACGCCGTCA-3'	cEt	N/A, control	N/A	N/A
549148	5'-GGCTACTACGCCGTCA-3'	cEt	N/A, control	N/A	N/A

Supplemental Figure 8