

Supplementary Table 1: Sequences recovered from each of the GANNAG selections.

See attached excel file (Supplementary Table 1.xlsx) for the sequences of the 2 finger modules recovered from each binding site selection.

Supplementary Table 2: Recognition helix sequences (positions 5 & 6 of Finger 1 and -1, 1 & 2 of Finger 2) of 16 two-finger modules that preferentially recognize each of the GANNAG target sites. Light blue background indicates modules where the indicated dinucleotide junction is not the most preferred sequence.

	A	C	G	T	3'
5'	A	KV QKP	KR QRN	RT QRS	
C	KT DPA	KR DPS	KR DKS	RT DHS	
G	KV RKG	KQ RNS	VR RFD	RT RSD	
T	KD ARG	RV HSS	KR LKY	QS FRT	

Supplementary Table 3. Sequences of the modularly-assembled and stitched ZFAs utilized in ZFNs in this study. (See attached file Supplementary Table 3.xlsx)

Supplementary Table 4: Comparison of modules used in ZFNs from the CoDA¹ study and our recent two finger module archives.

	CoDA ¹	gANNHg ²
Number of ZFNs assayed	39	18
Number of ZFNs active (>1%)	20	13
Percentage of GNN modules	99%	51%
Number of ZFNs with non-GNN fingers (number of active ZFNs)	2 (1)	18 (13)

1. Sander *et. al.*, *Nat. Methods* **8**, 67-69, 2011
2. ZFNs in this study and Gupta *et. al.*, *Nat. Methods* **9**, 588-90, 2012

Supplementary Table 5:

	Gupta 1/2FM	w/stitching
Archive Reference	A	B
Number of Unique ZFN sites in zebrafish protein-coding exons (25090 unique genes Zv9.64)	608,081	754,923
Fraction of zebrafish protein-coding genes containing ZFN site	95.0%	98.0%
Average density of ZFN sites (# bp/site)	132	109

A = Gupta, A. et al. *Nat. Methods* **9**, 588-90, 2012 & Zhu, C. et al. *Development* **138**, 4555, 2011.

B = Modules in Gupta 1/2FM archive and the new fingers and stitching method in this manuscript

Supplementary Figure 1. Recognition motifs for 77 GANNAG two-finger modules characterized in the B1H system. The recognition motif for each two-finger module is displayed as a Sequence Logo. The identity of the specificity determinants at the randomized recognition positions (Finger 1 positions 5 and 6, and Finger 2 positions -1, 1 and 2) in the characterized module are displayed above each recovered recognition motif.

Supplementary Figure 2. T7EI analysis of lesions in zebrafish targets for modularly-assembled ZFNs (*IRS2*, *met* and *sim1a*). Schematic representation of the 3 pairs of ZFNs targeting *IRS2*, *met* and *sim1a* are shown above each gel. Only the six finger constructs are shown. Finger number was reduced by progressively removing fingers from the N-terminus of the constructs. The positions of two-finger modules (GANNAG) described herein or fingers from other archives (GRNNCG (50) and single fingers (41)) within these ZFAs are indicated as in Figure 4, as is the position of the THPRAPIPKP linker that allows a single base pair to be skipped between intervening modules (65). T7EI digestion of PCR products spanning the genomic target site for normal embryos are shown. Cleavage products of the appropriate size for ZFN-induced lesions are indicated by red dots.

Supplementary Figure 3. B1H DNA-binding specificity for *abcc8* and *BRCA1* ZFAs. Sequence logos for each ZFA are shown with the dinucleotide junctions at the stitched interfaces boxed. The desired recognition sequence is shown to

the left of the logo with the dinucleotide junctions at the stitched interfaces underlined and the Adenines representing positions of two-finger module splice sites in bold. The recognition helices of the fingers are shown to the right of the logos with the C-terminal finger listed at the bottom. Stitched fingers are boxed in red.

Supplementary Figure 4. Top) Dose response curve of the *hebp2* ZFNs in zebrafish embryos. Morphology of embryos was assayed at 24 hpf (24). Bottom) Lesion frequency was assessed by T7EI sensitivity of a PCR product spanning the genomic target site (39,63). Lesion frequency was determined by ImageJ analysis of the uncleaved and cleaved DNA bands, where the black dot indicates the position of the cleaved band on the gel.

Supplementary Figure 5. Top) Dose response curve of the *abcc8* ZFNs in zebrafish embryos. Morphology of embryos was assayed at 24 hpf (24). Bottom) Lesion frequency was assessed by T7EI sensitivity of a PCR product spanning the genomic target site (39,63). Lesion frequency was determined by ImageJ analysis of the uncleaved and cleaved DNA bands, where the black dot indicates the position of the cleaved band on the gel.

Supplementary Figure 6. Top) Dose response curve of the *col17a1a* ZFNs in zebrafish embryos. Morphology of embryos was assayed at 24 hpf (24).

Bottom) Lesion frequency was assessed by Hpy166II resistance of a PCR product spanning the genomic target site. Lesion frequency was determined by ImageJ analysis of the uncleaved and cleaved DNA bands, where the red dot indicates the position of the uncleaved band on the gel.

Supplementary Figure 7. Lesion analysis for the *BRCA1* ZFNs in 293T cells. Lesion frequency was assessed by T7EI sensitivity of a PCR product spanning the genomic target site (39,63). Lesion frequency was determined by ImageJ analysis of the uncleaved and cleaved DNA bands, where the black dot indicates the position of the cleaved band on the gel.

Supplementary Figure 8. Lesion sequences recovered from zebrafish ZFN loci. Wild-type sequences are shown at the top of each set of sequence for each ZFN. Sequences with lesions recovered from our LacZ reporter system are shown below with the type of lesion indicated to the right of the sequence.

Supplementary Figure 1.

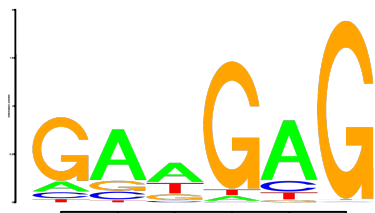
AQ-TGR



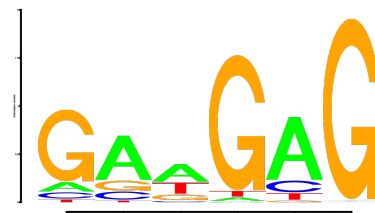
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AR-QKG



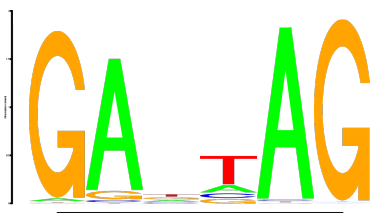
AR-QRG



AR-RGD



AT-SRS



GD-RAY



GR-TVH



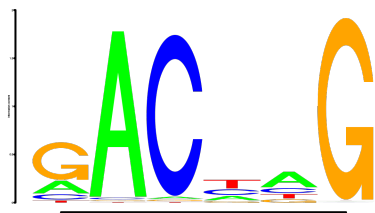
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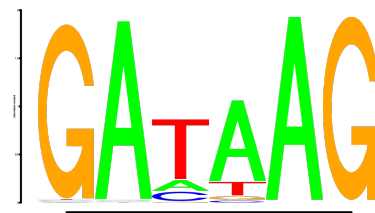
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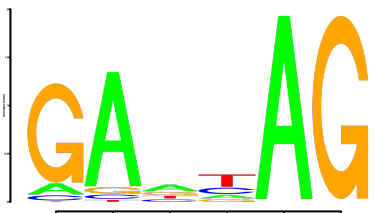
KA-DKS



KA-LSA



KA-QRS



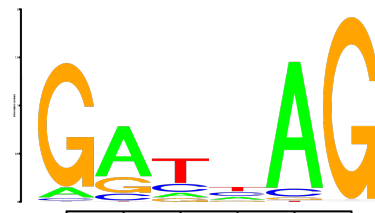
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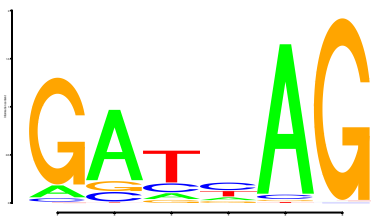
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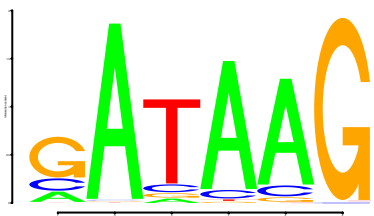
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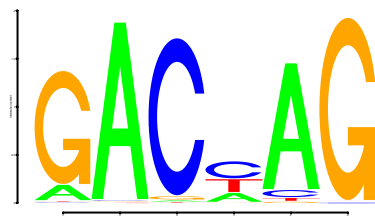
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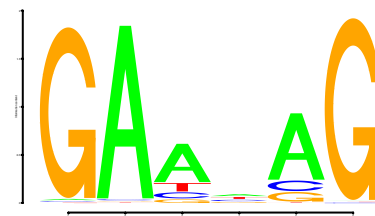
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KD-DRS



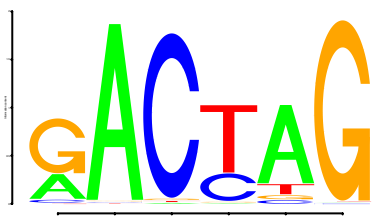
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KL-CRS



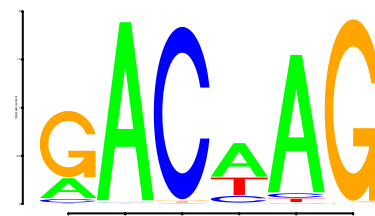
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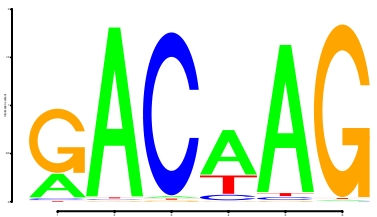
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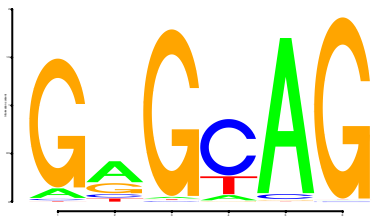
KN-DPA



KQ-DPA



KQ-RNS



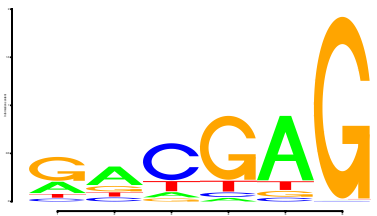
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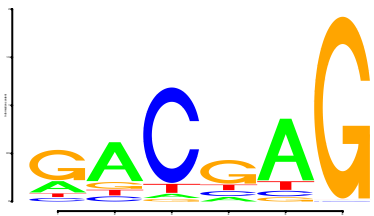
KQ-RSS



KR-DKS



KR-DPS



KR-ERG



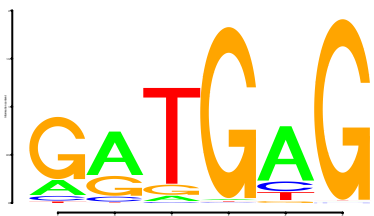
KR-LKY



LR-DPT



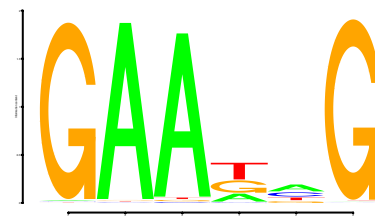
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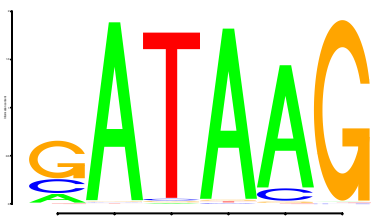
LR-TAY



LV-QSG



MD-ARG



MQ-RRF



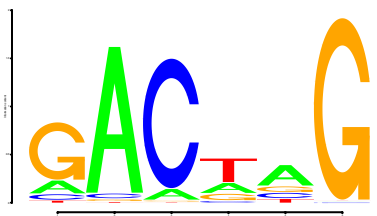
MR-RRC



MS-VKQ



NK-DPS



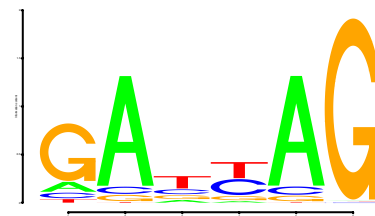
QA-FGG



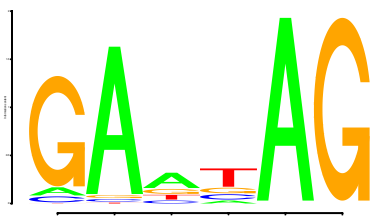
QS-FRT



QV-HSS



QV-QRS



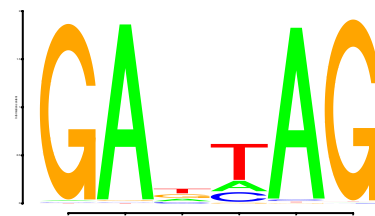
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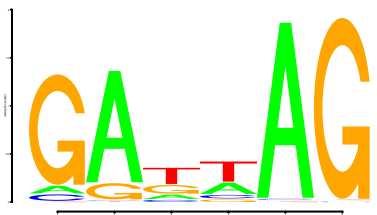
RR-TRY



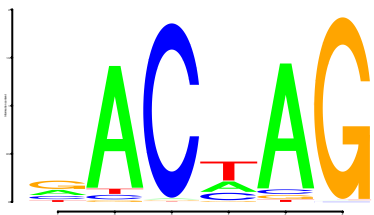
RS-AKS



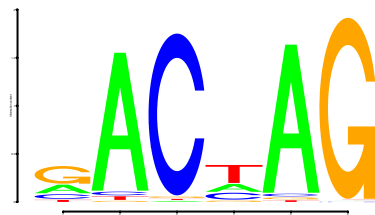
RT-ARS



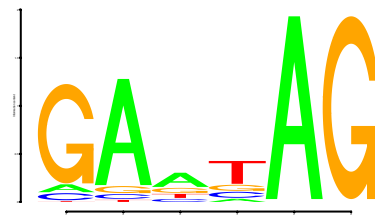
RT-DHS



RT-DRS



RT-QRS



RT-RID



RT-RSD



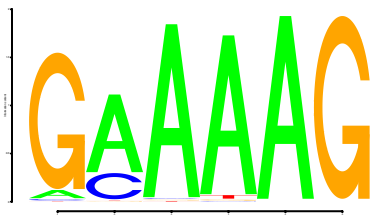
RT-TGS



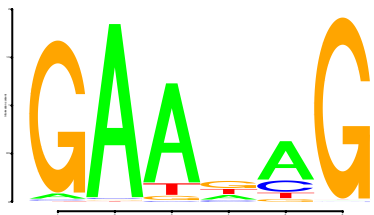
RV-HSS



RV-QLI



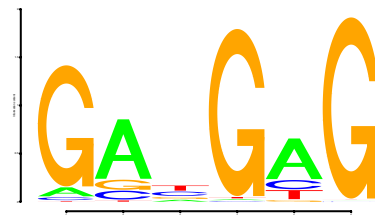
RV-QRG



VI-QSS



VR-CGS



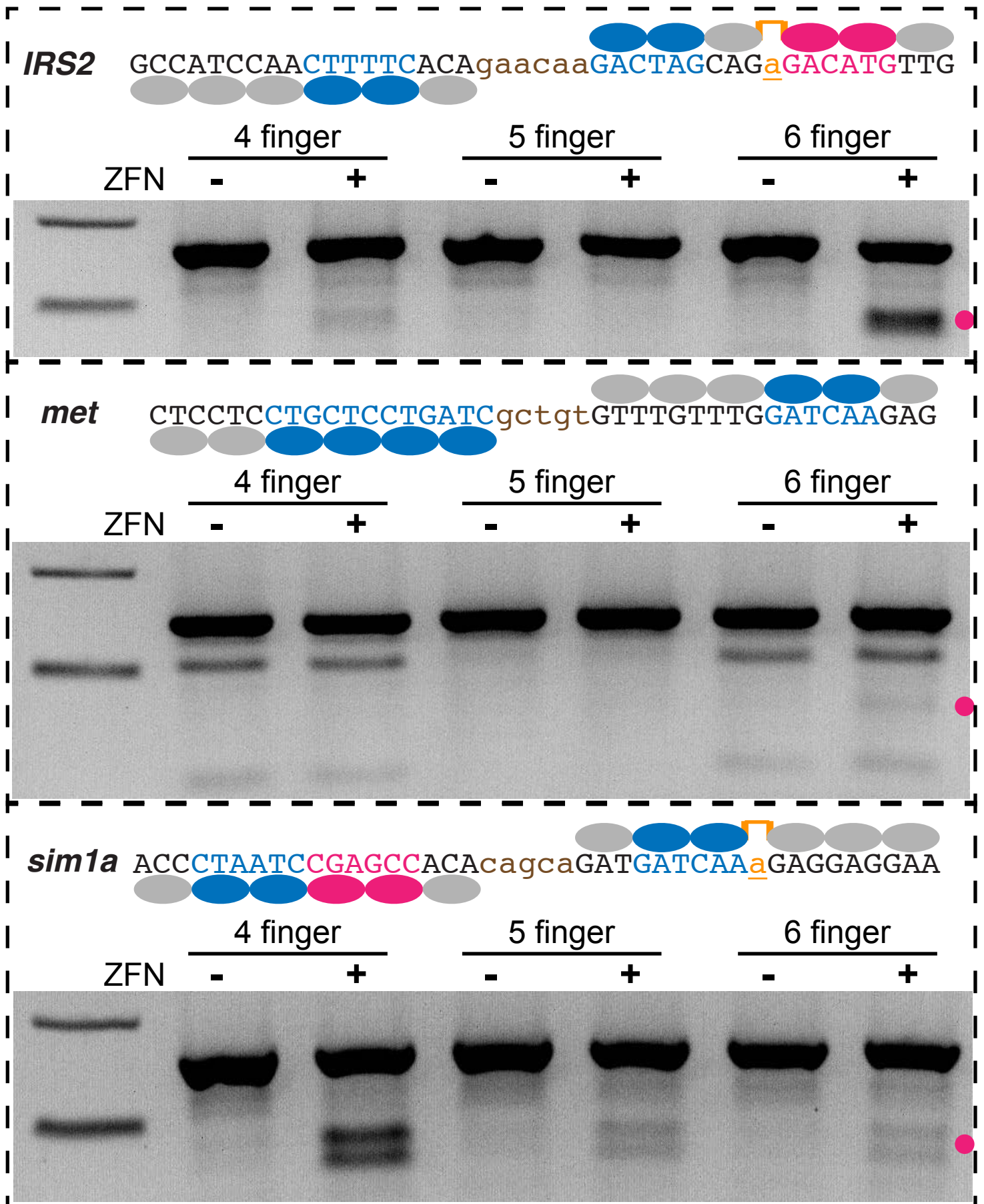
VR-RFD



Zif268



Supplementary Figure 2.



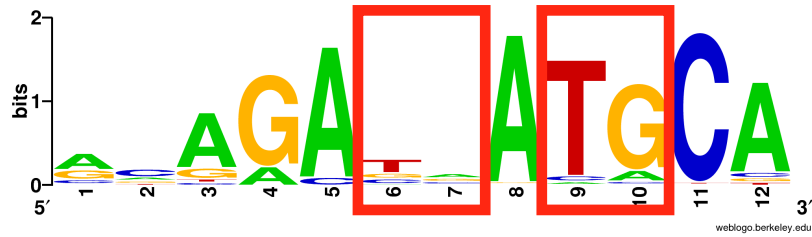
Supplementary Figure 3

abcc8-5p
 GAGAGCCG



F1 RSDTLKE
 F2 RSSNLKV
 F3 RKGNLTR

abcc8-3p
 ACAGATAATGCA



F1 QRGTLKR
 F2 LKYNLKQ
 F3 AAGNLTR
 F4 QRGTLKN

BRCA1-5p
 GACCACCACAG



F1 RSDNLSE
 F2 QRGTLVE
 F3 QRGNLKR
 F4 DPSNLTR

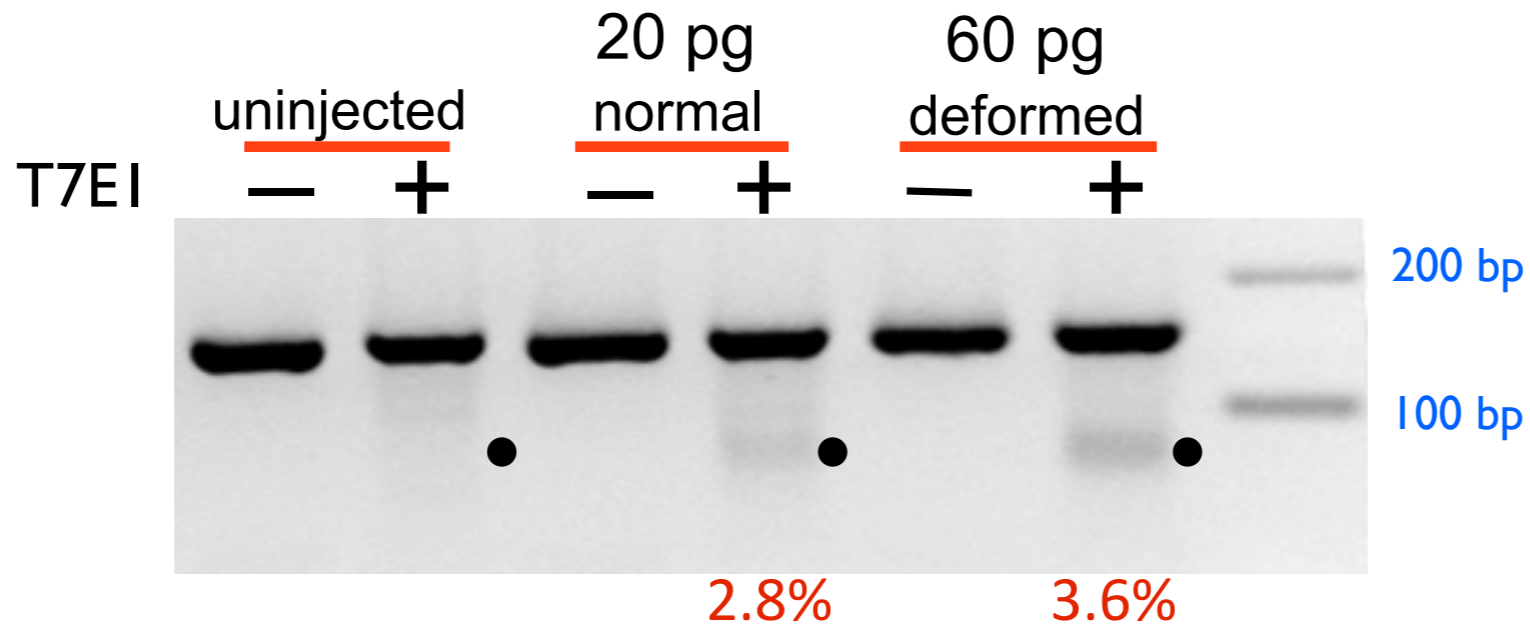
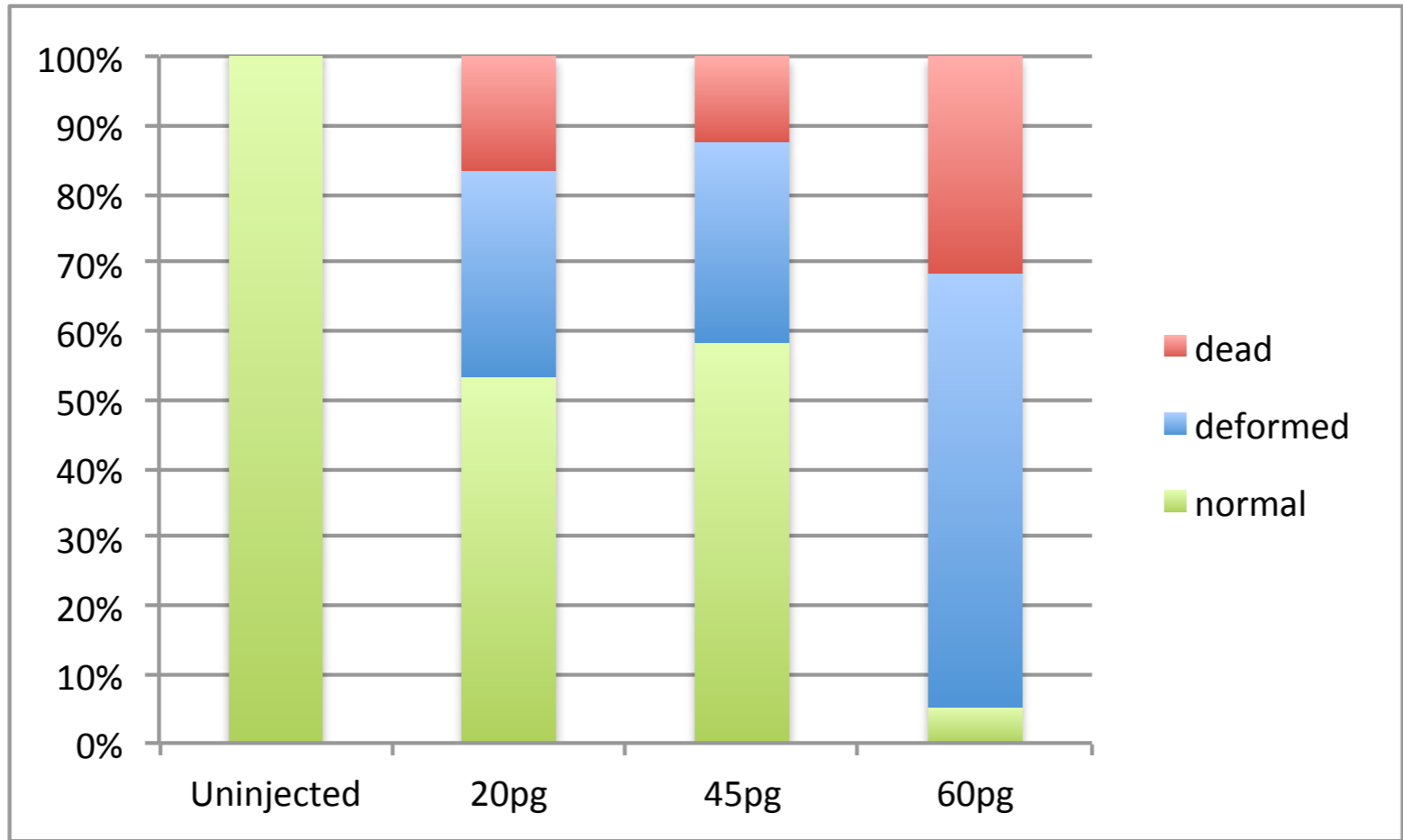
BRCA1-3p
 GATAAGCCAGTT



F1 TSGSLSR
 F2 QRGTLKE
 F3 RSSNLKQ
 F4 AAGNLTR

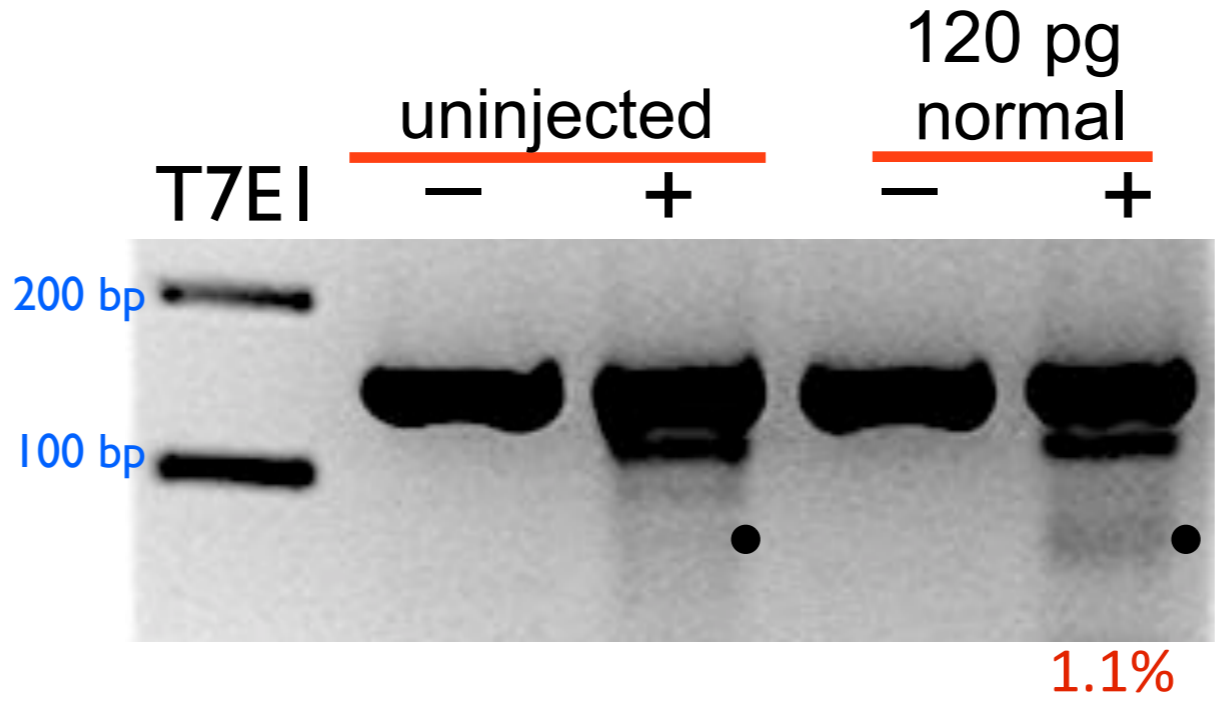
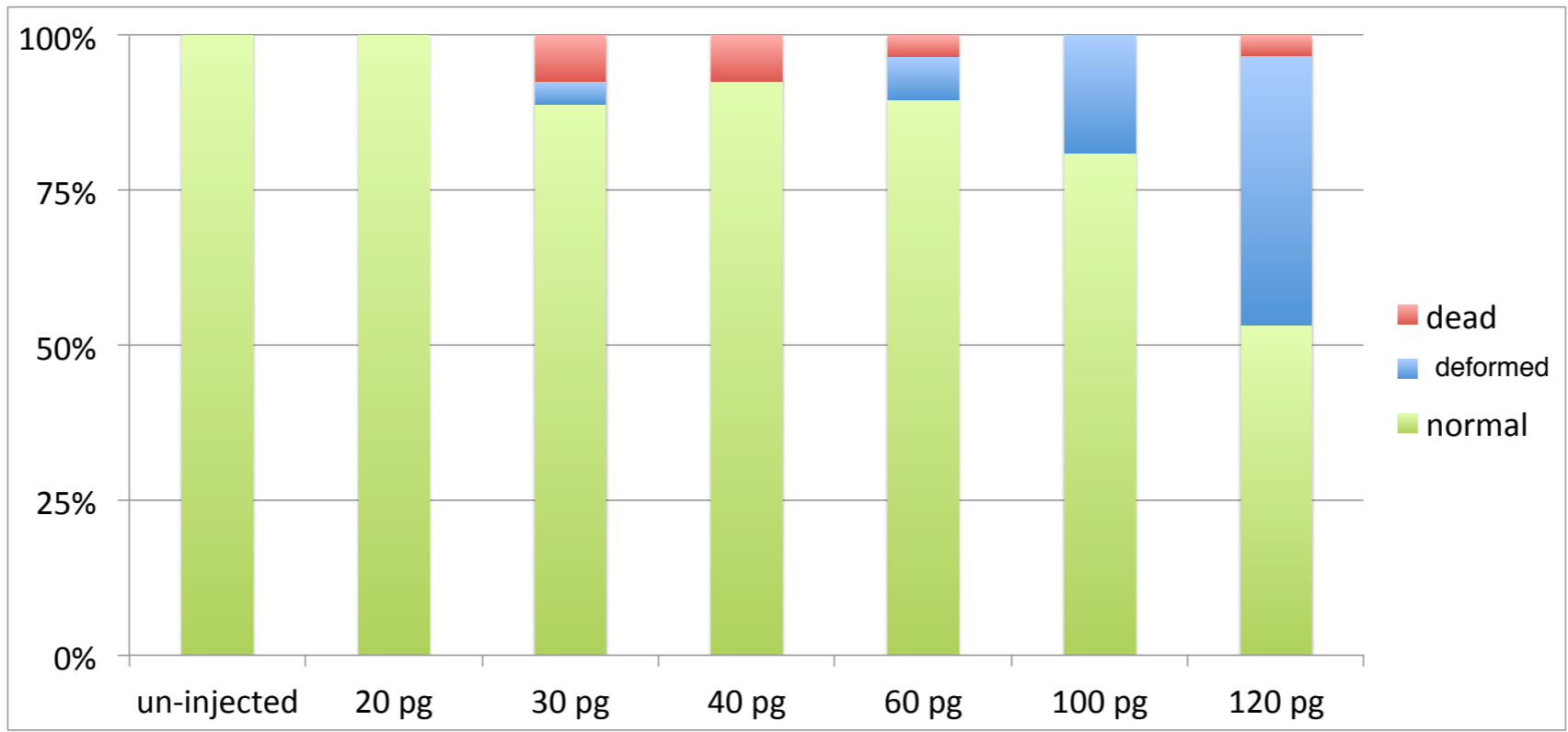
Supplementary Figure 4.

hebp2 **ATTGATACTTTTC** *agggt* **GATGACTATGAAG**



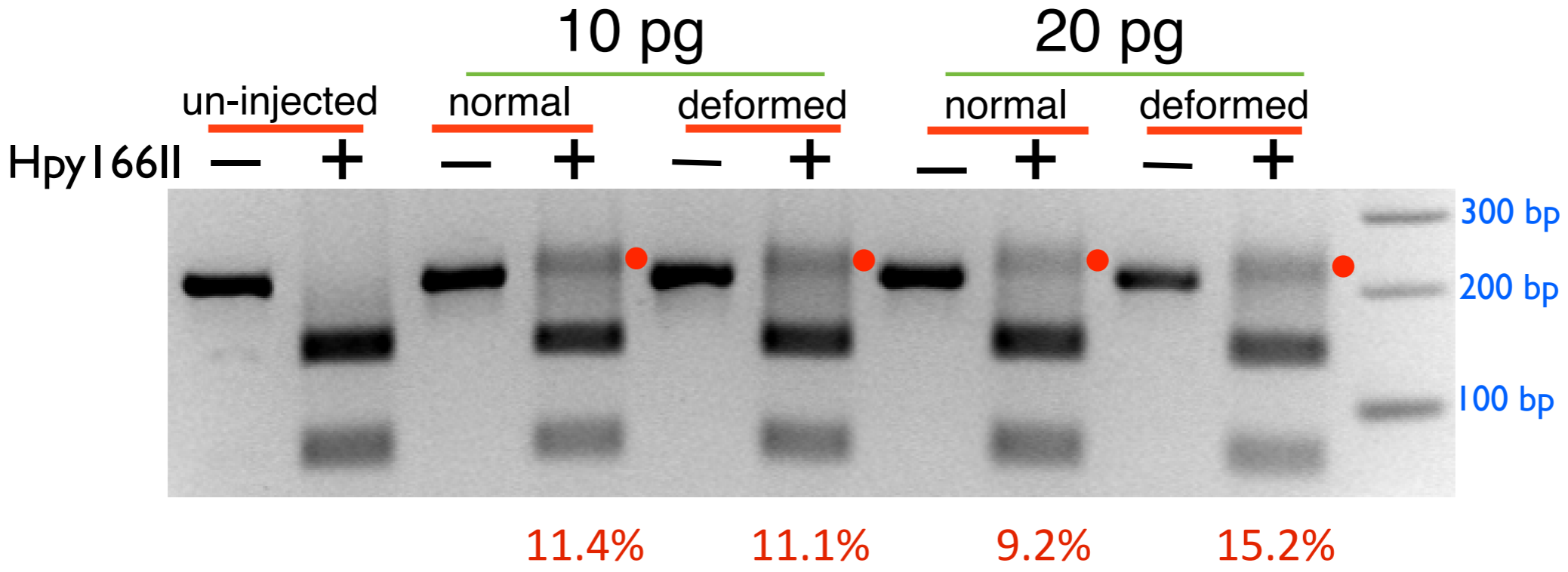
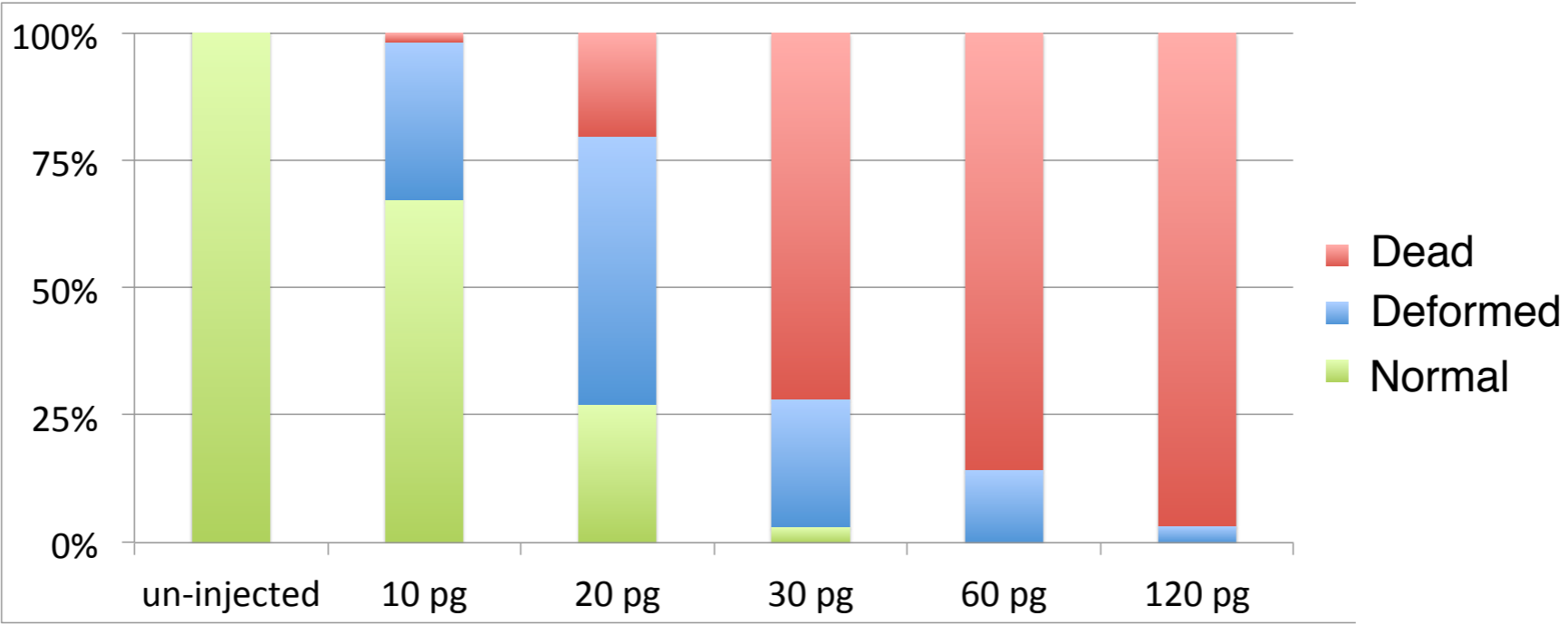
Supplementary Figure 5.

abcc8 CGGCTTCTC **tgtgg** ACAGATAATGCA



Supplementary Figure 6.

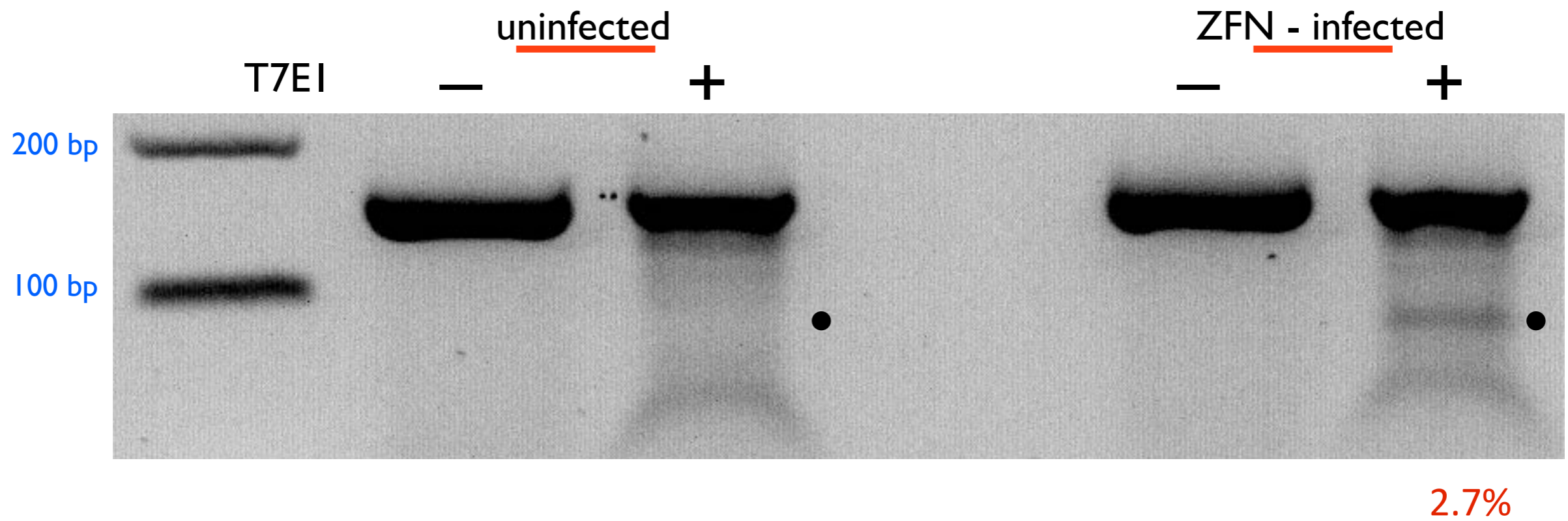
col17a1a GGTCCTGCTGGTC **cadctg** GACTATCAGGTAT
 HpyI66II



Supplementary Figure 7.

BRCA1

CTGTGGTTGGTC **agaaa** GATAAGCCAGTT



Supplementary Figure 8.

hebp2

GAA	TTGATACTTTTC	AG--GGT	GATGACTATGAAG	TCCG	Reference sequence
GAATTGATACTTT	-----	GGT	GATGACTATGAAGTCCG		-4
GAATTGATACTTTTCAG	-----	ATGACTATGAAGTCCG			-4
GAATTGATACTTTTC	-----	ATGACTATGAAGTCCG			-6
GAATTGATACTTTTC	-----	TATGAAGTCCG			-11
GAATTGATACTTTTC	-----	TATGACTATGAAGTCCG			-6, +1
GAATTGATACTT	-----	GTGGATGACTATGAAGTCCG			-8, +3
GAATTGATACTTTTCAG	--GT----	ACTATGAAGTCCG			-6, +1
GAATTGATACTTT	-GATACTTTGATGACTATGAAGTCCG				-6, +7
GAATTGATACTTTTCACTATGTGAAA	ACTATGAAGTCCG				-8, +10

IRS2

CAG	GCCATCCAAC	TTTTT	CACA	-----	GAACAA	GACTAGCAGAGACATGTTG	GAT	Reference sequence
CAGGCCATCCAAC	TTTTT	CACA	-----	CAGACTAGCAGAGACATGTTGGAT				-4
CAGGCCATCCAAC	TTTTT	CACA	-----	AGACTAGCAGAGACATGTTGGAT				-7
CAGGCCATCCAAC	TTTTT	CACA	-----	GCTAGCAGAGACATGTTGGAT				-7
CAGGCCATCCAAC	TTTTT	CAC	-----	TAGCAGAGACATGTTGGAT				-9
CAGGCCATCCAAC	TTTTT	CAC	-----	AGCAGAGACATGTTGGAT				-10
CAGGCCATCCAAC	TTTTT	CACA	-----	GCAGAGACATGTTGGAT				-11
CAGGCCATCCAAC	TTTTT	CACA	-----	GCAGAGACTAGCAGAGACATGTTGGAT				-4, +3
CAGGCCATCCAAC	TTTTT	CAC	-----	TTTTAGCAGAGACATGTTGGAT				-10, +3
CAGGCCATCCAAC	TTTTT	CACA	-----	GCAGACTAGCAGCAGACTAGCAGTAGACATGTTGGAT				-6, +14
CAGGCCATCCAAC	TTTTT	CACAGAGAAA	AAGCTTTTT	CACAGAGACTAGCAGAGACATGTTGGAT				-14, +23

coll7a1a

CAG	GCCTGCTGGTC	C	-----	ACCTG	GACTATCAGGTA	TCA	Reference sequence
CAGGCCTGCTGGT	-----	GGACTATCAGGTATCA					-6
CAGGCCTGCTGGTCC	-----	TATCAGGTATCA					-8
CAGGCCTGCTGGTC	-----	TATCAGGTATCA					-9
CAGGCCTGCTGGTCC	---TGGATAGGACTATCAGGTATCA						-4, +6
CAGGCCTGCTGGTCCACCT	-ACCTGGACTATCAGGTATCA						+4
CAGGCCT	---ATCAGGACTATCAGGACTATCAGGTATCA						-12, +13

abcc8

TAA	CGGCTTCTC	CGT---	GG	ACAGATAATGCA	CTG	Reference sequence
TAACGGCTTCTCCGTAACGGACAGATAATGCACTG						+3
TAACGGCT	---	CGT---	GGACAGATAATGCACTG			-4
TAACGGCTTCTC	-----	CAGATAATGCACTG				-6
TAACGGCTTCTC	-----	AGATAATGCACTG				-7
TAACGGCTT	-----	GATAATGCACTG				-11

sim1a

AGA	CCCTAATCCGAGCCACA	CAGCA	GATGATCAAAGAGGAG	GAAA	Reference sequence
AGACCCTAATCCGAGCCACA	--GCAGATGATCAAAGAGGAGGAAA				-2
AGACCCTAATCCGAGCCACA	-----	GATGATCAAAGAGGAGGAAA			-5

met

CTCCTCCTGCTCCTGATC	GCTGT	GTTTGTGGATCAAGAG	AAA	Reference sequence
CTCCTCCTGCTCCTGATG	-----	TTTGTGGATCAAGAGAAA		-6