APPENDIX

Clustering of strong replicators associated with active promoter are sufficient to establish an early-replicating domain

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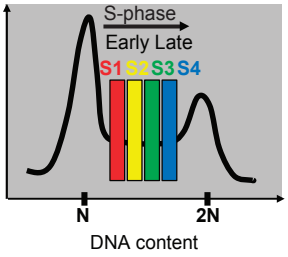
A

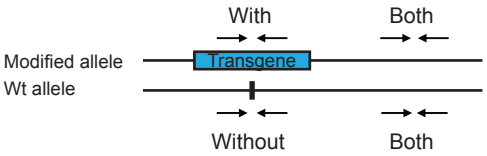
1) Asynchronous cells are BrdU pulse labelled for 1h

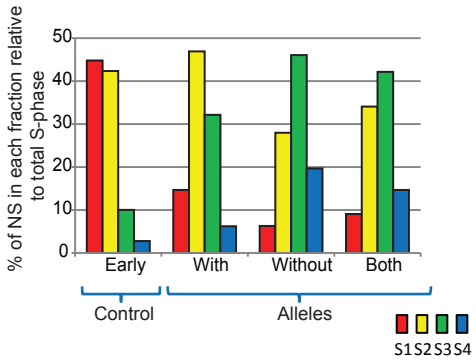
2) S-phase cells are sorted by flow cytometry into 4 fractions (S1 to S4)



3) The nascent BrdU-labelled DNA is enriched by immunoprecipitation

4) and quantified with allele specific primer pairs by qPCR analysis





B

Method 1:

Proportion differences between early fractions and late fractions

- ΔL= -27.3%
- ΔE= +8.4%
- -ΔL+ΔE = 35.7%

$\Delta L = [(\%S3+\%S4)_{with}] - [(\%S3+\%S4)_{without}]$

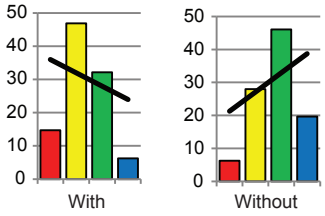
$\Delta E = [(\%S1)_{with}] - [(\%S1)_{without}]$

Global timing shift value: $-\Delta L+\Delta E$

C

Method 2:

Slope differences (Δslope) on four fractions between the modified and the Wt allele



Linear regression equations:

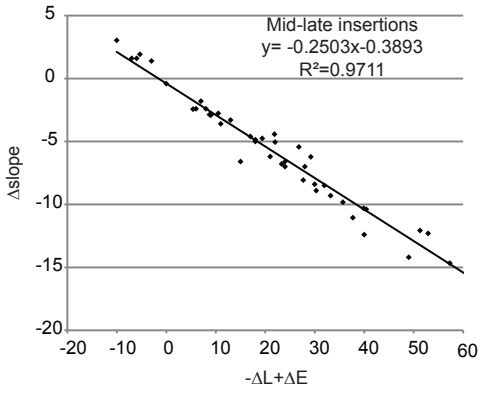
y=-4.0066x+35.016 y=5.8147x+10.463

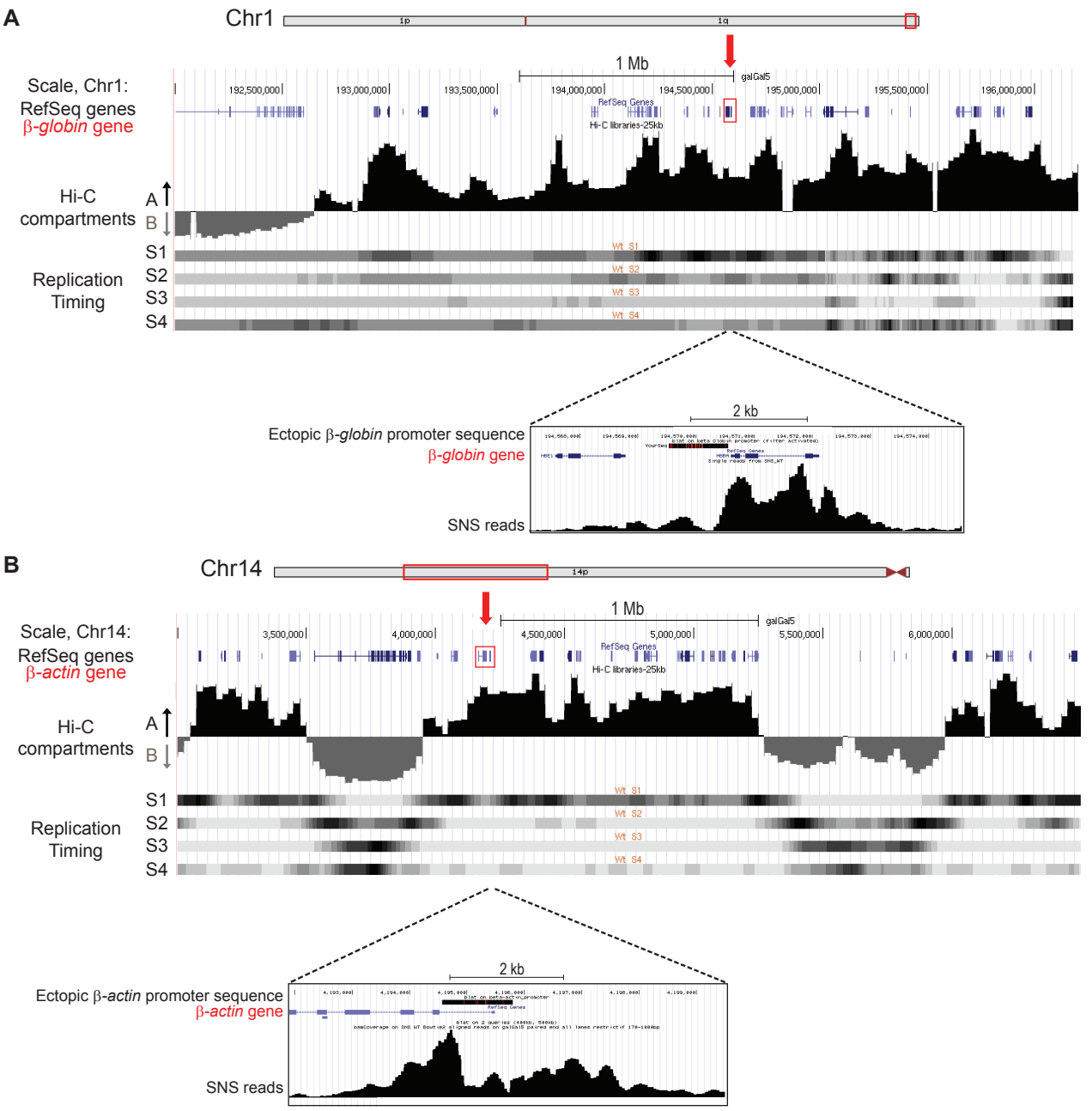
Δslope = -9.8

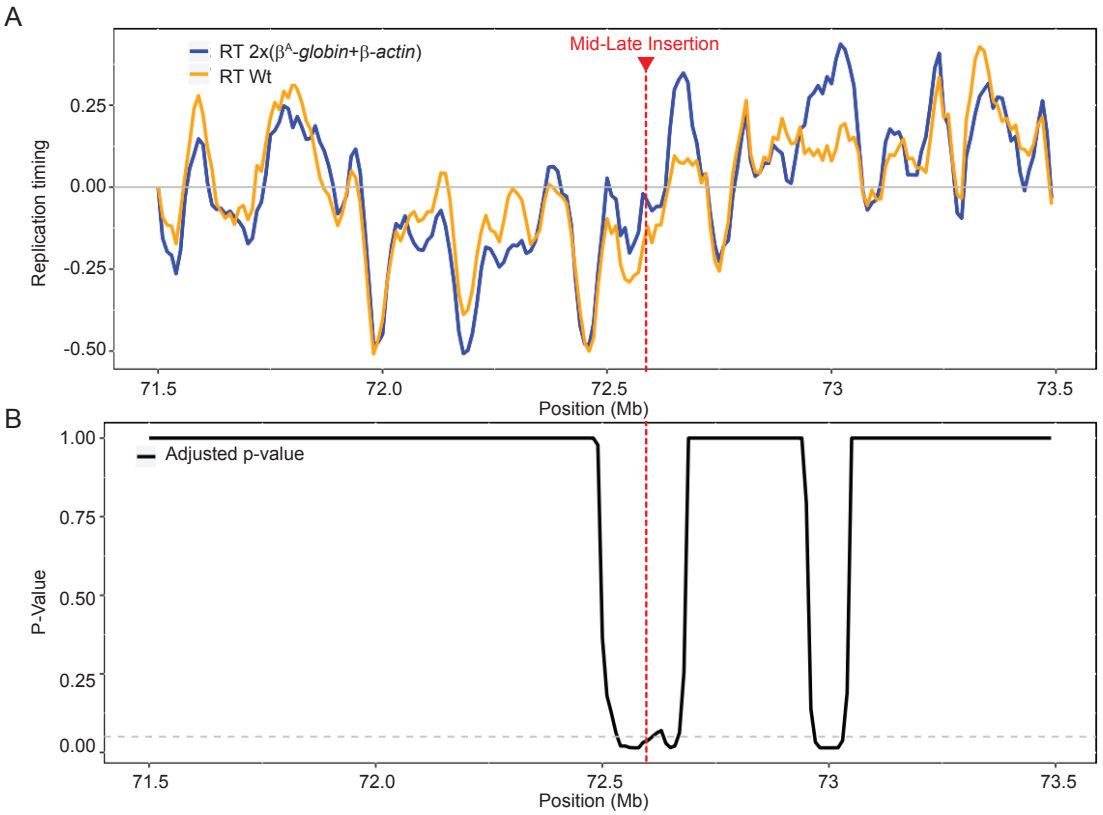
$\Delta slope = slope_{With} - slope_{Without}$

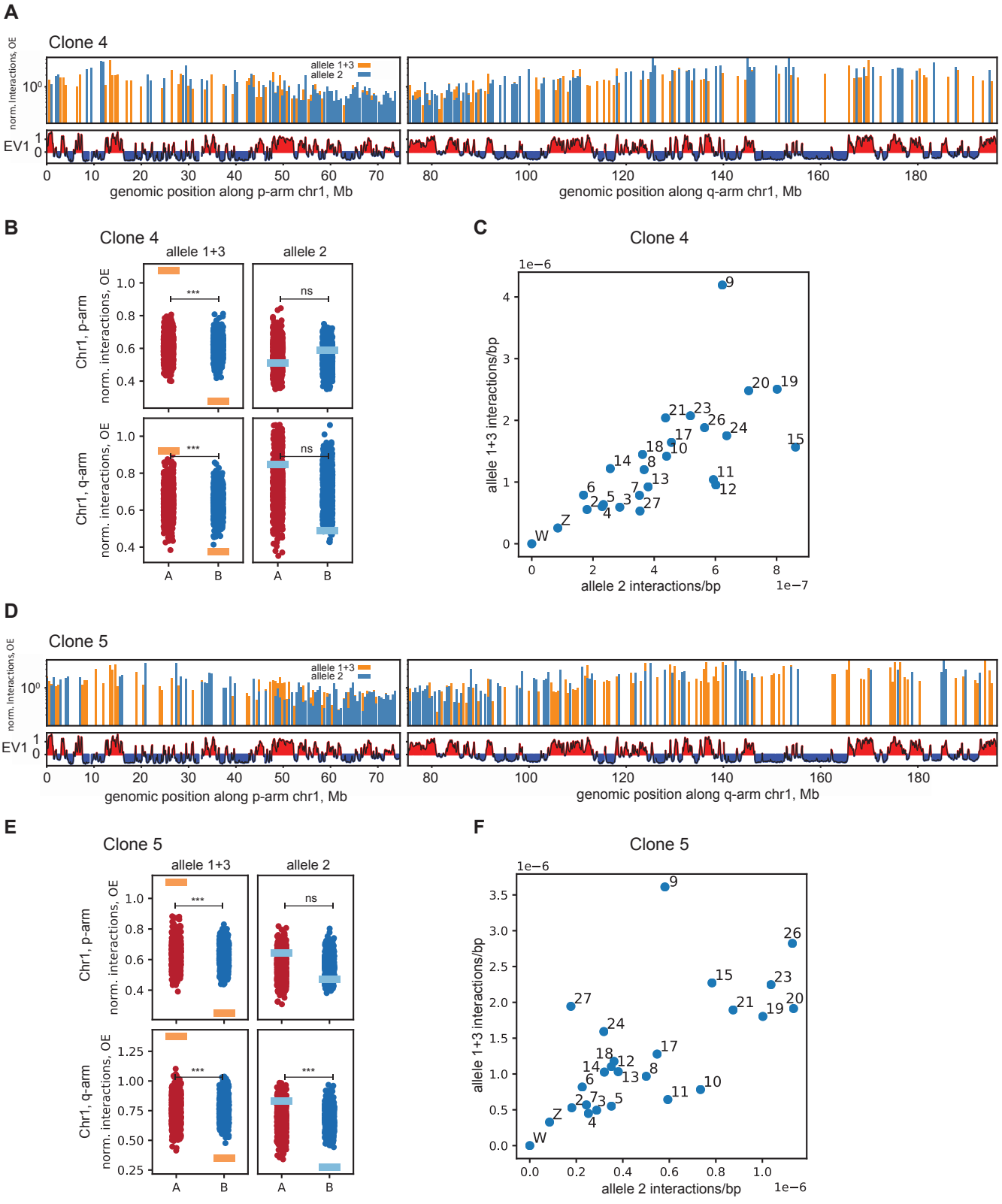
D

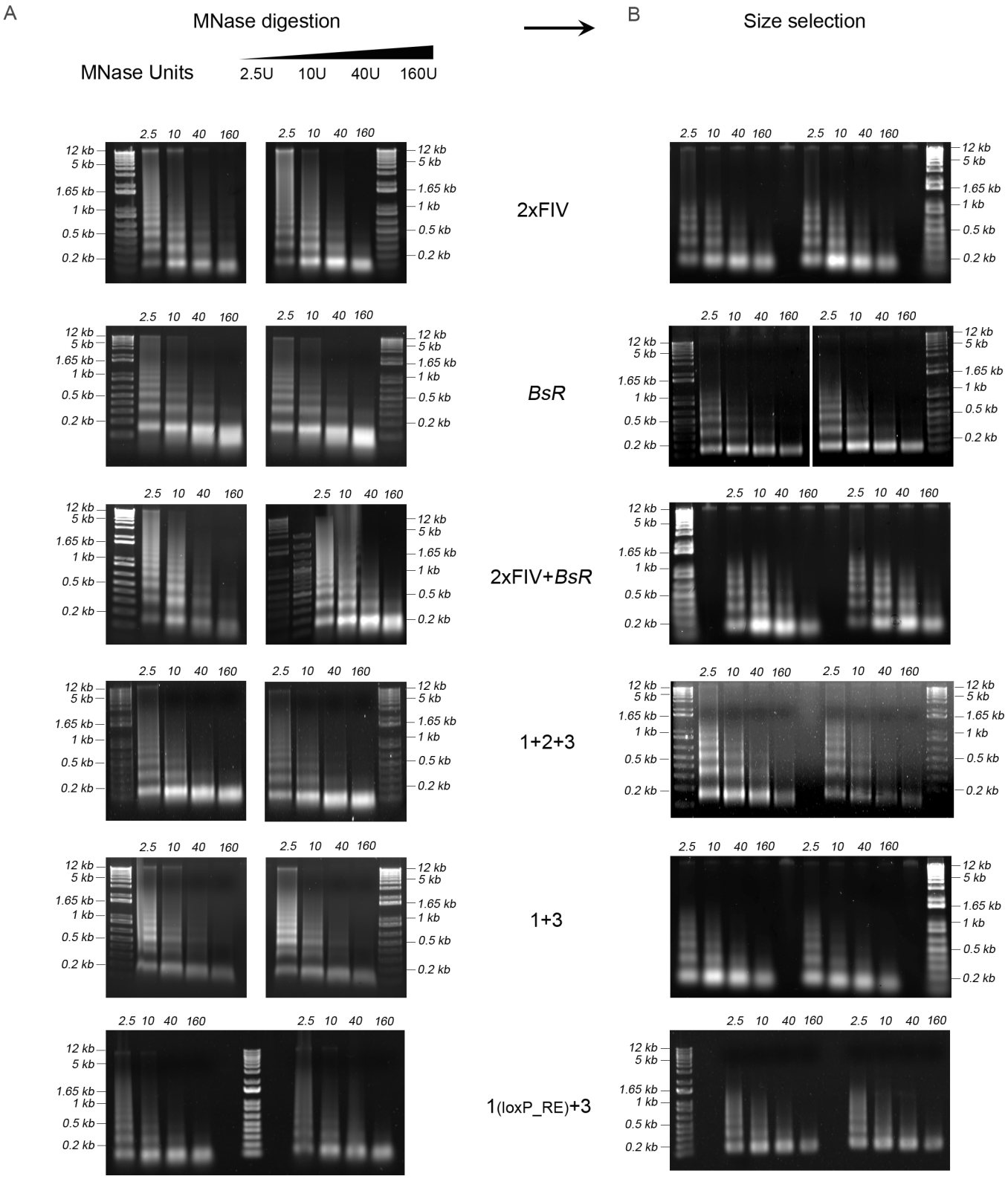
Correlation between the slope differences (Δslope) and the global timing shift value (-ΔL+ΔE)




A
Chr1
1 Mb
galGal5
Scale, Chr1:
RefSeq genes
β-globin gene
Hi-C compartments
A
B
Replication Timing
S1
S2
S3
S4
Hi-C libraries-25kb
2 kb
Ectopic β-globin promoter sequence
β-globin gene
SNS reads
B
Chr14
Scale, Chr14:
RefSeq genes
β-actin gene
Ectopic β-actin promoter sequence
β-actin gene

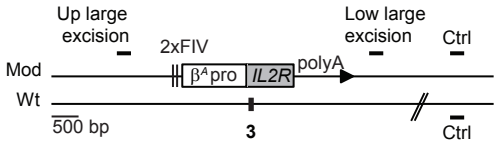

A
RT 2x(βA-globin+β-actin)
RT Wt
Mid-Late Insertion
Replication timing
0.25
0.00
-0.25
-0.50
71.5
72.0
72.5
73
73.5
Position (Mb)
B
Adjusted p-value
P-Value
1.00
0.75
0.50
0.25
0.00
71.5
72.0
72.5
73
73.5
Position (Mb)


A
Clone 4
norm. interactions, OE
allele 1+3
allele 2
EV1
genomic position along p-arm chr1, Mb
genomic position along q-arm chr1, Mb
B
Clone 4
allele 1+3
allele 2
Chr1, p-arm
Chr1, q-arm
norm. interactions, OE
ns
C
Clone 4
allele 1+3 interactions/bp
allele 2 interactions/bp
D
Clone 5
E
Clone 5
F
Clone 5

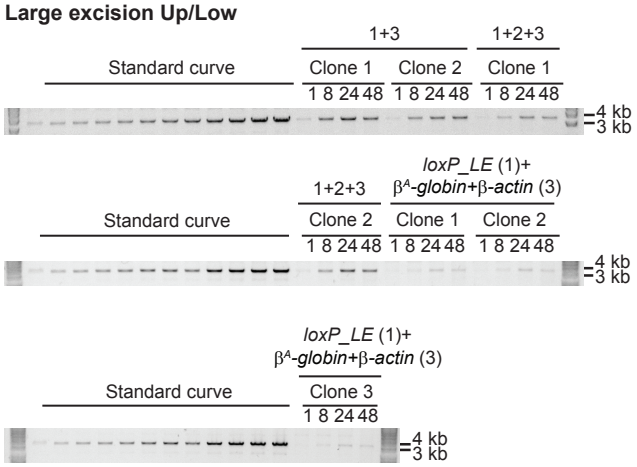

A
MNase digestion
MNase Units
2.5U
10U
40U
160U
B
Size selection
2.5 10 40 160
12 kb
5 kb
1.65 kb
1 kb
0.5 kb
0.2 kb
2xFIV
BsR
2xFIV+BsR
1+2+3
1+3
1(loxP_RE)+3

A

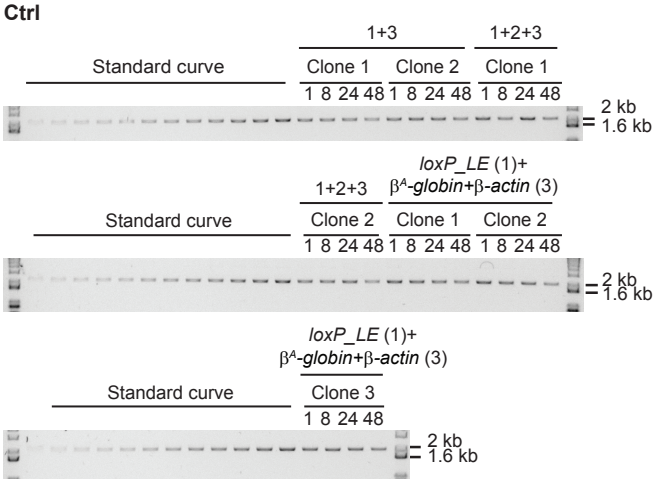
Large 1+3 and *loxP_LE* (1)+β^A-*globin*+β-*actin* (3) excision



Large excision Up/Low

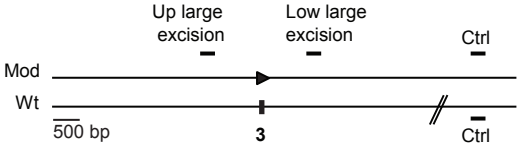


Ctrl

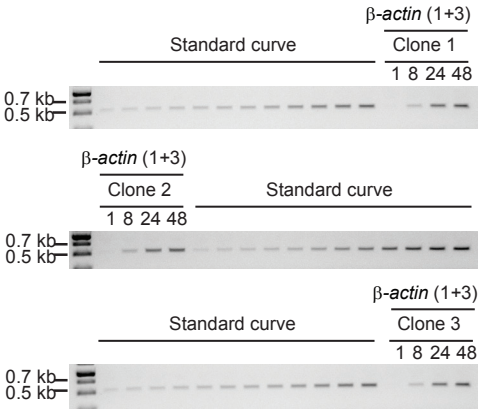


B

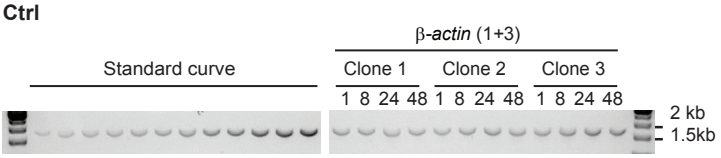
Large β-*actin* (1+3) excision

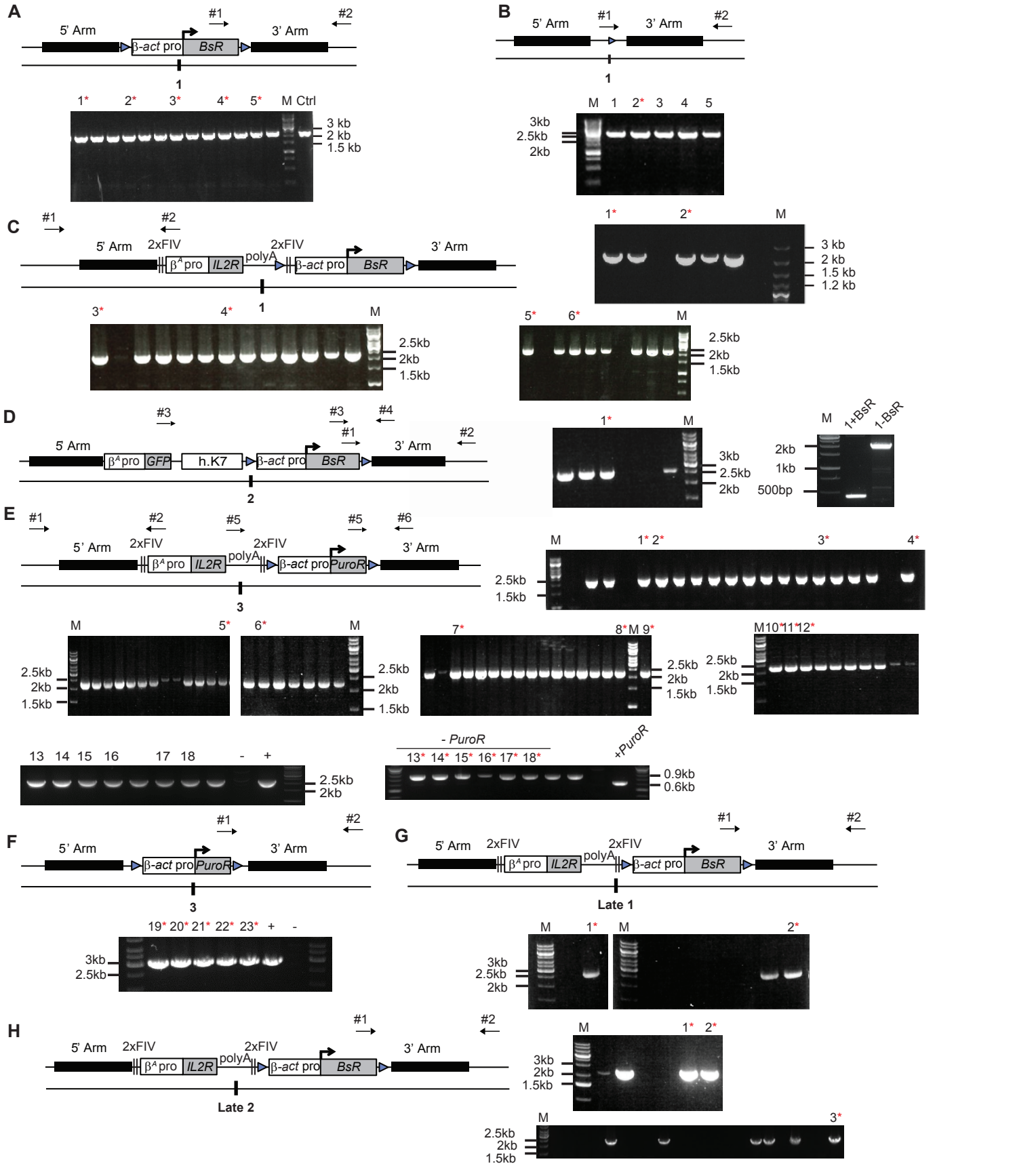


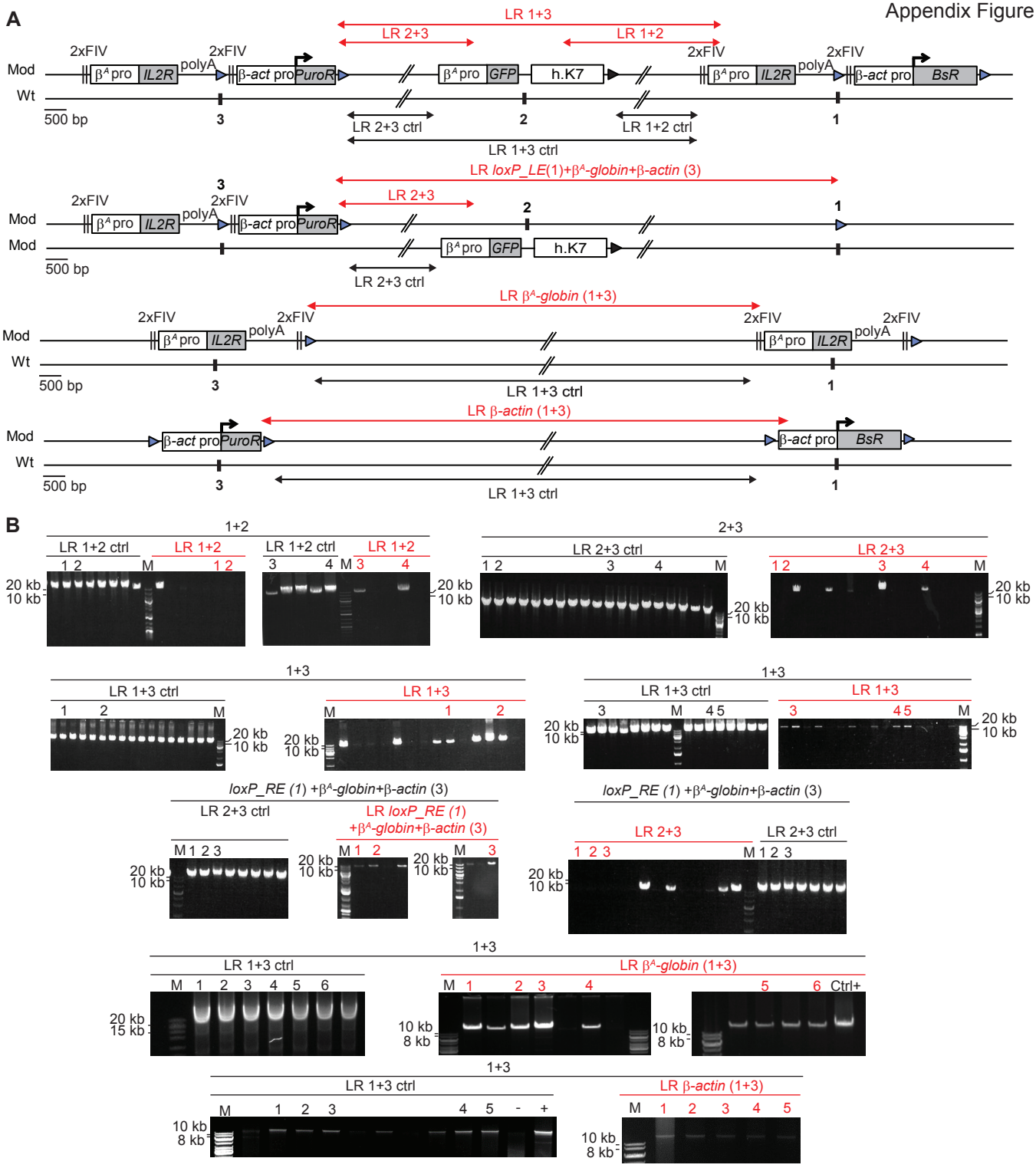
Large excision Up/Low

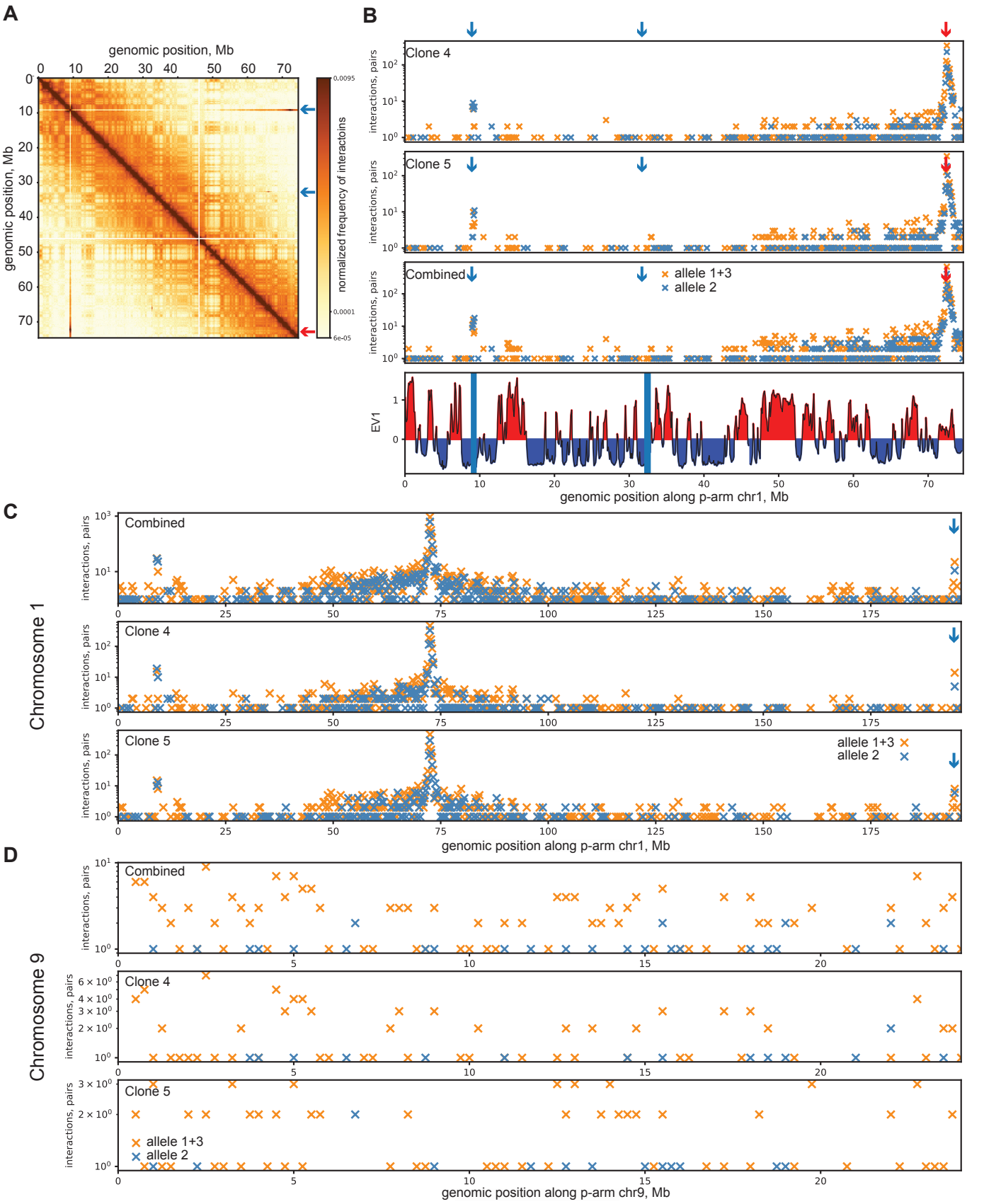


Ctrl

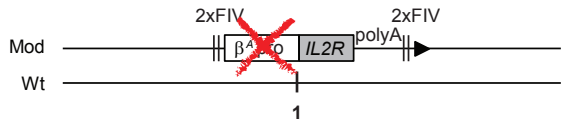
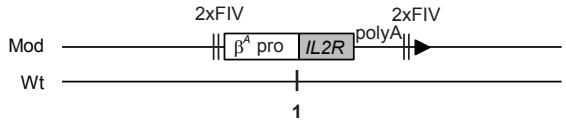
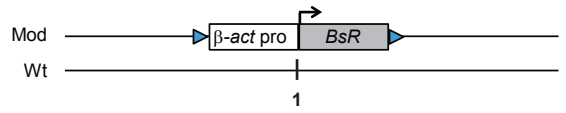
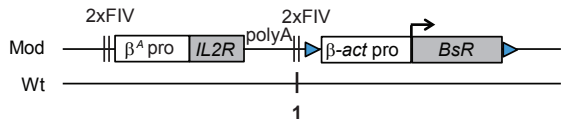
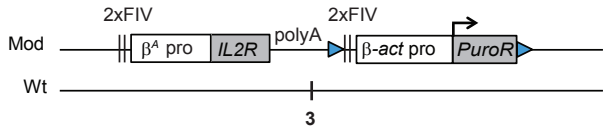
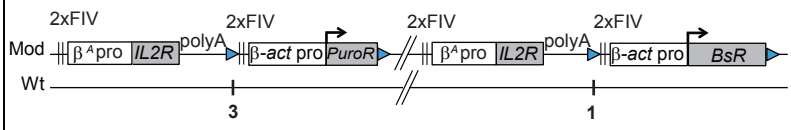
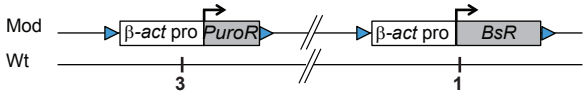
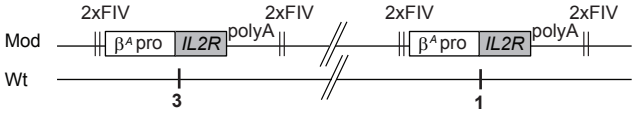
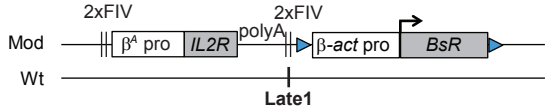
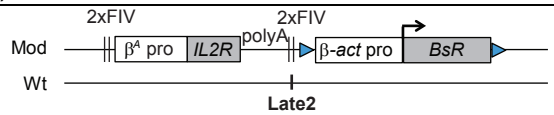



A
5' Arm
#1
#2
β-act pro
BsR
3' Arm
1
1* 2* 3* 4* 5* M Ctrl
3 kb
2 kb
1.5 kb
B
5' Arm
#1
3' Arm
#2
1
M 1 2* 3 4 5
3kb
2.5kb
2kb
C
#1
#2
5' Arm
2xFIV
β^A pro
IL2R
polyA
2xFIV
β-act pro
BsR
3' Arm
1
1* 2* M
3 kb
2 kb
1.5 kb
1.2 kb
3* 4* M
2.5kb
2kb
1.5kb
5* 6* M
2.5kb
2kb
1.5kb
D
#3
#3
#4
#1
#2
5 Arm
β^A pro
GFP
h.K7
β-act pro
BsR
3' Arm
2
1* M
3kb
2.5kb
2kb
M 1+BsR 1-BsR
2kb
1kb
500bp
E
#1
#2
#5
#5
#6
5' Arm
2xFIV
β^A pro
IL2R
polyA
2xFIV
β-act pro
PuroR
3' Arm
3
M 1* 2* 3* 4*
2.5kb
1.5kb
M 5* 6* M
2.5kb
2kb
1.5kb
7* 8* M 9*
2.5kb
2kb
1.5kb
M 10* 11* 12*
2.5kb
2kb
1.5kb
13 14 15 16 17 18 - +
2.5kb
2kb
- PuroR
13* 14* 15* 16* 17* 18*
+PuroR
0.9kb
0.6kb
F
#1
#2
5' Arm
β-act pro
PuroR
3' Arm
3
19* 20* 21* 22* 23* + -
3kb
2.5kb
G
#1
#2
5' Arm
2xFIV
β^A pro
IL2R
polyA
2xFIV
β-act pro
BsR
3' Arm
Late 1
M 1* M 2*
3kb
2.5kb
2kb
H
#1
#2
5' Arm
2xFIV
β^A pro
IL2R
polyA
2xFIV
β-act pro
BsR
3' Arm
Late 2
M 1* 2*
3kb
2kb
1.5kb
M 3*
2.5kb
2kb
1.5kb


A
LR 1+3
LR 2+3
LR 1+2
2xFIV
polyA
Mod
Wt
βA pro
IL2R
β-act pro
PuroR
GFP
h.K7
BsR
500 bp
LR 2+3 ctrl
LR 1+2 ctrl
LR 1+3 ctrl
LR loxP_LE(1)+βA-globin+β-actin (3)
LR βA-globin (1+3)
LR β-actin (1+3)
B
1+2
2+3
1+3
LR 1+2 ctrl
LR 1+2
LR 2+3 ctrl
LR 2+3
LR 1+3 ctrl
LR 1+3
loxP_RE (1) +βA-globin+β-actin (3)
LR 2+3 ctrl
LR loxP_RE (1) +βA-globin+β-actin (3)
LR 2+3
LR 2+3 ctrl
LR 1+3 ctrl
LR βA-globin (1+3)
LR 1+3 ctrl
LR β-actin (1+3)
20 kb
10 kb
15 kb
8 kb
Ctrl+


A
genomic position, Mb
genomic position, Mb
normalized frequency of interactoins
B
Clone 4
Clone 5
Combined
allele 1+3
allele 2
interactions, pairs
EV1
genomic position along p-arm chr1, Mb
C
Chromosome 1
Combined
Clone 4
Clone 5
allele 1+3
allele 2
interactions, pairs
genomic position along p-arm chr1, Mb
D
Chromosome 9
Combined
Clone 4
Clone 5
allele 1+3
allele 2
interactions, pairs
genomic position along p-arm chr9, Mb

Appendix Table S1

Site of insertion	Number of clones and source	RT shift Median values
Mid-Late (1)	Total= 44	
	10 { 2 Hassan-Zadeh et al. 2012 (Figure 8); 8 Valton et al. 2014 (Figure 8C, S10) }	-ΔL+ΔE= +6.5%
	8 { 2 Hassan-Zadeh et al. 2012 (Figure 6B); 6 Valton et al. 2014 (Figure 8C, S9) }	-ΔL+ΔE= +20.2%
	6 { 1 Hassan-Zadeh et al. 2012 (Figure 4A); 5 This study (Figure EV1A.) }	-ΔL+ΔE= +19.6%
	8 { 2 Hassan-Zadeh et al. 2012 (Figure 6A); 6 This study (Figure EV1B.,EV2B-C.) }	-ΔL+ΔE= +36.6%
Mid-Late (3)		
	3 { This study (Figure EV2D-E.) }	-ΔL+ΔE= +26.8%
Mid-Late (1+3 and 1+2+3)		
	5 { This study (Figure EV3A-B.) }	-ΔL+ΔE= +65.2%
	5 { This study (Figure EV3C.) }	-ΔL+ΔE= +38.5%
	7 { This study (Figure EV5) }	-ΔL+ΔE= +11%
Late (1)		
	2 { This study (Figure 8A.) }	-ΔL+ΔE= +29.3% / +51.7%
Late (2)		
	3 { This study (Figure 8B.) }	-ΔL+ΔE= +19.1%

	Construction tested / site	Both concentration	With concentration	Ratio With/Both
Insertions at site mid-1				
β-*actin* (1)	β-*actin* / 1	0.97	0.45	0.47
β-*actin* (2)		0.78	0.32	0.41
β-*actin* (3)		0.63	0.37	0.59
β-*actin* (4)		0.97	0.62	0.63
β-*actin* (5)		0.93	0.41	0.44
β^A-*globin*+β-*actin* (1)	β^A-*globin*+β-*actin* / 1	1.34	0.71	0.53
β^A-*globin*+β-*actin* (2)		1.29	0.65	0.5
β^A*GFP* h.K7+β^A-*globin*+β-*actin* (1)		0.81	0.45	0.55
β^A*GFP* h.K7+β^A-*globin*+β-*actin* (2)		0.84	0.43	0.51
β^A*GFP* h.K7+β^A-*globin*+β-*actin* (3)		0.74	0.4	0.54
β^A*GFP* h.K7+β^A-*globin*+β-*actin* (4)		0.91	0.45	0.49
LoxP_LE (1)	LoxP_LE / 1	0.58	0.28	0.48
Insertions at site mid-2				
β^A*GFP* h.K7 (1)	β^AGFP h.K7 / 2	1.12	0.54	0.49
Insertions at site mid-3				
β^A-*globin*+β-*actin* + β^A*GFP* h.K7 (1)	β^A-*globin*+β-*actin* / 3	1.04	0.57	0.55
β^A-*globin*+β-*actin* + β^A*GFP* h.K7 (2)		1.59	0.59	0.37
β^A-*globin*+β-*actin* + β^A*GFP* h.K7 (3)		1.08	0.43	0.4
Insertions at site mid-1 and mid-3				
β^A-*globin*+β-*actin* + β^A*GFP* h.K7+β^A-*globin*+β-*actin* (1)	β^A-*globin*+β-*actin* / 1 and 3	0.56	0.73	1.29
β^A-*globin*+β-*actin* + β^A*GFP* h.K7+β^A-*globin*+β-*actin* (2)		0.54	0.58	1.07
β^A-*globin*+β-*actin* + β^A*GFP* h.K7+β^A-*globin*+β-*actin* (3)		1.15	1.33	1.16
β^A-*globin*+β-*actin* + β^A*GFP* h.K7+β^A-*globin*+β-*actin* (4)		0.87	0.99	1.13
β^A-*globin*+β-*actin* + β^A*GFP* h.K7+β^A-*globin*+β-*actin* (5)		0.83	0.94	1.13
β^A-*globin*+β-*actin* +LoxP_LE (1)	LoxP_LE / 1	3.34	2.21	0.66
β^A-*globin*+β-*actin* +LoxP_LE (2)		2.19	1.39	0.63
β^A-*globin*+β-*actin* +LoxP_LE (3)		2.12	1.13	0.53
β-*actin* (1+3) (1)	β-*actin* / 3	1.17	0.71	0.61
β-*actin* (1+3) (2)		1.23	0.78	0.64
β-*actin* (1+3) (3)		1.22	0.7	0.57
β-*actin* (1+3) (4)		0.95	0.52	0.55
β-*actin* (1+3) (5)		1.04	0.57	0.54
β^A-*globin* (1+3) (1)	β^A-*globin* / 1 and 3	0.65	0.63	0.97
β^A-*globin* (1+3) (2)		0.74	0.62	0.83
β^A-*globin* (1+3) (3)		0.92	1.2	1.31
β^A-*globin* (1+3) (4)		0.77	1.0	1.30
β^A-*globin* (1+3) (5)		0.75	0.66	0.88
β^A-*globin* (1+3) (6)		0.2	0.22	1.1
Insertions at site late-1				
β^A-*globin*+β-*actin* (1)	β^A-*globin*+β-*actin* / late 1	2.15	0.96	0.45
β^A-*globin*+β-*actin* (2)		1.71	0.75	0.44
Insertions at site late-2				
β^A-*globin*+β-*actin* (1)	β^A-*globin*+β-*actin* / late 2	1.6	0.59	0.37
β^A-*globin*+β-*actin* (2)		1.37	0.5	0.36
β^A-*globin*+β-*actin* (3)		0.98	0.43	0.44

Appendix Table S3

Primer name	Primer sequence	Insertion site	galGal5 Assembly	Amplicon size
5'arm_ML1	(Hassan-Zadeh et al., 2012)	site mid-late 1	chr1:72,565,520	
3'arm_ML1				
5'arm_ML2_Up	CCAAACCAGGCCACTCTTAGT	site mid-late 2	chr1: 72,548,589	2,163 bp
5'arm_ML2_Low	AGTCACTTGGCATAAATAAGAAGCC			
3'arm_ML2_Up	CTGAGCAGGAAGGGAAACGA			2,068 bp
3'arm_ML2_Low	CCATAGTGCAGACCTGGCAT			
5'arm_ML3_Up	ACACACTCACCTCCTGCCTT	site mid-late 3	chr1: 72,536,060	2,054 bp
5'arm_ML3_Low	AGATCTCAGTCCTGCCAGCA			
3'arm_ML3_Up	AAGTTCGTAATACACAACCTTGAC			2,062 bp
3'arm_ML3_Low	GCTTCCCCGCTTCTTCCCTA			
5'arm_L1_Up	CAGTGAAACACAGGAGGAACA	site late 1	chr1:70,523,649	2,244 bp
5'arm_L1_Low	TAACTCCAAGAACGATCACTGC			
3'arm_L1_Up	GGAAATGTCTTGAATCTCACAAGG			2,137 bp
3'arm_L1_Low	ATGCCCACCAGTGTCCATAA			
5'arm_L2_Up	ACTTGTTGAGCCTTTATGGAGAAC	site late 2	chr1:177,936,192	1996 bp
5'arm_L2_Low	CGGTGTTACAGAGGAGTAAACTGA			
3'arm_L2_Up	ACCTTATGCATTTCGTTCCTATGT			2001 bp
3'arm_L2_Low	TAAGAAGAGAGATGGGGATCAAAC			
2XFIV_β^{A}*globin*-Up	GGGGACAACTTTGTATACAAAAGTTGAGGTGGCACGGGATCGCTTTCCTAGGTGGCACGGGATCGCTTTCCTCTGCCCACACCCTCCTG			
2XFIV_β^{A}*globin*-Low	GGGGACAACTTTGTATAGAAAAGTTGGGTGGGTACCACTAGTGATGATCCGTCATCCAGACATG			
2XFIV_β-*actin*-Up	GCTCGAGAGGTGGCACGGGATCGCTTTCCTAGGTGGCACGGGATCGCTTTCCTGTGAGC CCCACGTTCTGCTT			
2XFIV_β-*actin*-Low	CTTCTCTGTCGCTACTTCTAC			
XhoI+2xFIV-Up	GACTCTCGAGAGGAAAGCGATCCCGTGCCACCTAGGAAAGCGATCCCGTGCCACCTGCTAGCCCTGATCAATAACT			
XhoI+2xFIV-Low	AAGTCCTCGAGCGTATTACAATTCACTGGCCGT			
β^{A}-Up	CTGCCCACACCCTCCTG			
β^{A}-Low	TTCCTGACCCTTGGGACCA			
GFP-Up	GGGTCAGGAAATGGTGAGCAAGGGCGAGG			
GFP-Low	TGAAGCAGCATTTACGCCTTAAG			
h.K7-Up	TGCTGCTTCATTTCTGCTCTC			
h.K7-Low	GCAGAGCCAGAGTCCAAGAG			
loxP_RE-h.K7-Up	GGATCCATAACTTCGTATAGCATACATTATACGAACGGTAACTTGAGCCCAGGAGTTCGA			
loxP_RE-h.K7-rev	AGAGTTCCAACCCCAGCCTC			

Appendix Table S4

	Forward primer sequence	Reverse primer sequence	Genomic position (Build Dec 2015)
Replication timing analysis			
With on *GFP* reporter	GGAATTCGATAGCTTGGCGGC	GCTGAACTTGTGGCCGTTTAC	
With on insertion site 2	GTAATGAAATTCAGCAATGACAGGC	TCCTATCTGTTCAAATGTGCATCAG	chr1:72548543+72548676
With on β^A-*globin*+β-*actin*	GGGGACTGCTCACGTTCATCA	AATGTGGCGTGTGGGATCTC	
With on β-*actin*	TGCAGAAATCGGAGGAAGAAGA	GAATTGCCGCTCCCACATGA	
Without or Wt allele insertion site 1	CAGGACAGCAGGTATTCACA	GGCCTGAACACTGTGTCAAT	chr1:72565497+72565651
Without or Wt allele insertion site 2	GTAATGAAATTCAGCAATGACAGGC	TCCTATCTGTTCAAATGTGCATCAG	chr1:72548543+72548676
Without on *GFP* reporter site 2	GGAATTCGATAGCTTGGCGGC	GCTGAACTTGTGGCCGTTTAC	
Wt allele insertion site 3	TGGTACAGGCTGAGGACACC	TGATGACTGCAGCTTCCTTCT	chr1:72535996+72536105
Without or Wt allele insertion site late1	CCCTTGAATCAGACCCTTGA	CCCTCCTTTCTCCATAAAAACA	chr1:70523547+70523674
Without or Wt allele insertion site late 2	TTTACACTACTCCCACCCCTCG	TTGACCATATGCCACCAACACC	chr1:177936339+177936438
Controls			
Both or 1+ 5 kb	TCCATACAGCCACAACAGCA	TGTGGAAGAGTTTCAGTCCAGG	chr1:72570952+72571067
Both or late 1- 4.8 kb	TGTACTTCTCTGTGGACATGCA	TGGCACAGAGGACAGGTAAGA	chr1:70518727+70518810
Both or late 2 - 3.6kb	CAAGGTTTCCACCCCTAAAGA	TGATGGATGTGGGAAGAGAAA	chr1:177932452+ 177932533
Early timing control	GACGGTCAGGTTTGCCAAAG	TCCTGAGGATACGTTTTTCAG	chr1: 194563998+194564262
Mitochondrial DNA	CATCCCATGCATAACTCCTG	GTAGTCCAGGCTTCACTTGA	chrM :541+731
Chlps, Chromatin accessibility and RNA quantification			
5' 2xFIV-1	GGGCTATTCAGCTTGTCTAG	GCCACCTCAACTTTTGTATAC	
5' 2xFIV-3	TTATGCTGGCAGGACTGAGA	GTGGGCAGAGGAAAGCGAT	
β^A pro 1	GGGAGCAAGAGCCCAGAC	GTGAGCAGTCCCCACATCAG	
β^A pro 2	GGGACTGCTCACGTTCATCA	AATGTGGCGTGTGGGATCTC	
IL2R gene	CTACACAGAGGTCCTGCTG	GTGAAGAGAAGCCTCAGGCA	
3' 2xFIV-1	TGCATTCTAGTTGTGGTTTGTCC	ACCGTCGACCAACTTTGTATAGA	
3' 2xFIV 1-3	AAGCTTGGATCCCCTACCGT	GAGAGTGAAGCAGAACGTGGG	
5' β-*actin* pro	GTGGGACTTCTTCTAAAGGGCTA	TGATCAATAACTTCGTATAATGTATGCT	
β-*actin* pro	TGCAGAAATCGGAGGAAGAAGA	GAATTGCCGCTCCCACATGA	
BsR gene	CGGCAGTACATATTGAAGCGT	CCCTACACATACCACAAGGA	
PuroR gene	ACGACCTTCCATGACCGAGT	AGTTCTTGCAGCTCGGTGAC	
β^A-*GFP*	GGAATTCGATAGCTTGGCGGC	GCTGAACTTGTGGCCGTTTAC	
GFP gene	GCCCGACAACCACTACCTGAG	GCTTTACTTGTACAGCTCGTCCA	
h.K7	AAGTTTATCATTGTGTGGCAGTCA	GTTCTGGTTGGGTTGTATGTCAC	
LoxP site	CGCCCTATAGTGAGTCGTATTACA	TGTTTAAATGTTTGTAGCCTGCCT	
cond1	CATCTGTGCTCTGGGTCCA	AAGGAGTGGAAGGAACGCATC	chr1:194546368+194546497
cond 2	TTGGTGCAGTGCCTCAGATAG	ATGTCGCTTGTCACGATGGAT	chr1:194546457+194546563
MED 14 pro	GGATTCACACTGTTCCCCTCC	TGCATGTTTCTCTCATCCGAAGT	chr1:112227330+112227461
BU1A pro	CTCTGTAGCCAGATCGTCTTCTC	GTGTCAGCTCATCTAGGCAAATC	chr1:91922377+91922546
BU1A gene	AATGTCCCCAAAATGAGCTG	CCTCTTTTTCCACCCTCCTC	chr1:91923373+91923517
MED 14 gene	TGGGCTAATAATGCTGGAAAGGT	TAGAGAAGCCAGACGATCAGCA	chr1:112236082+112236823
Long-range amplifications			
LR 1+2	AAGGGTCAGCTTTCGTGATAATCTGG	ACCTCTCTTGCATTCACAGTTCAACA	
LR 1+2 ctrl	GCAAGATGGGCAGAGCTGAGTTAACAAT	TGTCCTGTAAGTCCTGGCAAAACAAAGA	chr1:72548824+72565503
LR 2+3	GAGCGTATTACAATTCACTGGCCGTC	CTTGCTCACCATTTCCTGACCCTTG	
LR 2+3 ctrl	GCAGTATAACAAGCACGCCTGAAGTAAA	CAGTCTTATCCCACCCCTTCCTGATAG	chr1:72536307+72548324
LR 1+3	GAGCGTATTACAATTCACTGGCCGTC	GATCCCGTGCCACCTCAACTTTTGTAT	
LR loxP LE(1)+ β^A-*globin*+β-*actin* (3)	GAGCGTATTACAATTCACTGGCCGTC	GACGTTGTGGCTGTTGTAGTTGTACTC	
LR β^A-*globin* (1+3)	TCCAATTCGCCCTATAGTGAGTCGTA	GATCCCGTGCCACCTCAACTTTTGTAT	
LR β-*actin* (1+3)	CTCCAATTCGCCCTATAGTGAGTCGTAT	GATACCGTCGACCAACTTTGTATAGAAAA	
LR 1+3 ctrl	GCAGTATAACAAGCACGCCTGAAGTAAA	TGTCCTGTAAGTCCTGGCAAAACAAAGA	chr1:72597486+72626682
Screening of targeted integration			
3'-screening-site 2 for *GFP* reporter	GCTCCAATTCGCCCTATAGTGA	GGCACTCCATTTCCATCTCCT	
5'-screening-site 3 for β^A-*globin*+β-*actin*	GGACTGGCTAGGGAACAAGAG	TCTGCCTTCTCCCTGATAACG	
5'-screening-site 1 for β^A-*globin*+β-*actin*	GTGCAGCATCAGTGGATAAAGT	TCTGCCTTCTCCCTGATAACG	
3'-screening-site 1 for β-*actin*	CCCCCTGAACCTGAAACATAA	CCACATGTTTATTGCATACGGC	
3'-screening site 1 for *loxP LE*	CCAATTCGCCCTATAGTGAGTCG	ACGTAACAAATCTACAGGTCTTCG	
3'-screening site 3 for *PuroR*	CCCCCTGAACCTGAAACATAA	CTTCCAACCCAGGCCACTATG	
3'-screening site late 1 for β^A-*globin*+β-*actin*	GAGCTCCAATTCGCCCTAT	ACTATTGTACCCCTCCCTGTTG	
3'-screening site late 2 for β^A-*globin*+β-*actin*	GAGCTCCAATTCGCCCTAT	GGTCTGATCCCTATCTCACTGG	
Screening of site specific excision			
BsR excision at site 2	CCCCCTGAACCTGAAACATAA	TATCCCACTGCCACTGGAGG	
PuroR excision at site 3	TGCATTCTAGTTGTGGTTTGTCC	GGTCTGAGAGGTCTTGCTGGA	
Large 1+3 excision Up/Low	GGTTCTGGTGCCCTCATTGAT	TGCTCTGCTAGAATTGCCTGT	chr1:72535815+72565623
Ctrl	ACCCAAGGCAGGCTACAAAC	TGAGTTACTTTGGCATTACTTTTCATC	chr1:72565577+72567483
No *PuroR* excision	CCCCCTGAACCTGAAACATAA	GCAGCCTTTTCCAGCAAGAC	
No *BsR* excision	ACGACCTTCCATGACCGAGT	AGTTCTTGCAGCTCGGTGAC	
PuroR excision	CACGCCACATTCAAAGCCATG	TCCAGCAAGACCTCTCAGACC	
BsR excision	CAGCGGAGACAGAGGAAGAGT	GGCATGGTTTTGATTTCTGGCC	
Copy number quantification			
loxP LE	CGCCCTATAGTGAGTCGTATTACA	TGTTTAAATGTTTGTAGCCTGCCT	
PuroR	ACGACCTTCCATGACCGAGT	AGTTCTTGCAGCTCGGTGAC	

name	length	GC	DpnII sites/bp	sample	total interactions	cis percent
allele1+3	2532	56.7	0.0043	clone 4	2433	67.41
allele1+3	2532	56.7	0.0043	clone 5	2369	69.78
allele2	2509	46.2	0.0032	clone 4	1318	80.2
allele2	2509	46.2	0.0032	clone 5	1290	78.53
chr1	196202544	40	0.0021	clone 4	121564119	81.08
chr1	196202544	40	0.0021	clone 5	116517761	80.63

Appendix Figure S1: Quantitative analysis of RT shifts by two distinct calculation methods reveals cooperation between two combinations of *cis*-regulatory elements

A. Method for allele-specific analysis of RT by real-time PCR quantification. BrdU pulse-labeled cells were sorted into four S-phase fractions (S1 to S4) and the immunoprecipitated newly synthesized strands (NS) were quantified by real-time qPCR in each fraction. Specific primer pairs determine the RT profile for the modified allele (With), the wt allele (Without) and both alleles (Both). The endogenous *β-globin* locus was analysed as an early-replicated control.

B-C. Two methods of calculation were used to determine the difference in RT between the wt and modified alleles: the -ΔL+ΔE method and the ΔSlope method.

D. The analysis of the correlation between the ΔSlope and the -ΔL+ΔE method for all clonal cell lines previously published (Hassan-Zadeh et al., 2012; Valton et al., 2014) or analysed for the first time here is shown. The linear regression curve is shown, with the corresponding equation and the coefficient of determination (R^2).

Appendix Figure S2: Endogenous genomic features of early-replicated domains containing the *β-globin* and *β-actin* loci

A-B. UCSC genome browser visualization (galGal5) of 4 Mb genomic windows inside either the *β-globin* locus of chromosome 1 or the *β-actin* locus of chromosome 14 are shown. Annotated genes are represented and the β^A*-globin* and the *β-actin* genes are indicated by a red arrow. Track of eigenvector 1 values corresponding to A/B compartments at 25 kb resolution after Hi-C analysis are represented. Tracks of nascent strands (NS) enrichments obtained after cells sorting into four S-phase fractions, BrdU pulse-labeled nascent strands (NS) immunoprecipitation, and sequencing were represented separately (S1 to S4) for the wt cell line in dense configuration. Below, an enlargement of either the β^A*-globin* or the *β-actin* promoter region with the ectopic sequence found in our constructs and the single reads from SNS aligned previously published (Massip et al., 2019) are reported.

Appendix Figure S3: P-value distribution for the effect of the *β^A-globin + β-actin* construct on RT in the mid-late locus

A. Replication timings of the WT and the 2x(*β^A-globin* + *β-actin*) insert were smoothed using 500kb sliding windows, then centered and normalized so that the variability of timings were comparable between the two conditions.

B. A t-test was performed for each window to assess the significance of the timing differences. Adjusted p-values were computed for each window and smoothed using the PLIS R-package (Wei et al., 2009) to account for their spatial genomic dependency. P-values were then adjusted by the Benjamini-Hochberg procedure to ensure the control of the False Discovery Rate. The dotted line corresponds to a FDR of 5%.

Appendix Figure S4: Interaction profiles of allele 1+3/2 with chromosome 1 for clones 4 and 5

A. Interaction profiles of allele 1+3/2 with chr1 for clone 4 Hi-C libraries: distance-corrected interactions, *i.e.* observed/expected (OE), binned at 500 kb with corresponding compartment (EV1) tracks.

B. OE interactions of allele 1+3 (orange rectangles) and allele 2 (blue rectangles) averaged over A and B compartments for p and q-arms at 100kb bin size for clone 4. Values corresponding to OE interactions of allele 1+3 and allele 2 averaged over shuffled A (red circles, N=1000) and B compartments (blue circles, N=1000) for p and q-arms. ns, not significant; *p<0.05; **p<0.01;***p<0.001.

C. Scatterplot of density of interactions between allele 1+3 and allele 2 with chromosomes larger than 5,000,000 bp for clone 4.

D. Interaction profiles of allele 1+3/2 with chr1 for clone 5 Hi-C libraries : distance-corrected interactions, *i.e.* observed/expected (OE), binned at 500 kb with corresponding compartment (EV1) tracks.

E. OE interactions of allele 1+3 (orange rectangles) and allele 2 (blue rectangles) averaged over A and B compartments for p and q-arms at 100kb bin size for clone 5. Values corresponding to OE interactions of allele 1+3 and allele 2 averaged over shuffled A (red circles, N=1000) and B compartments (blue circles, N=1000) for p and q-arms. ns, not significant; *p<0.05; **p<0.01;***p<0.001.

F. Scatterplot of density of interactions between allele 1+3 and allele 2 with chromosomes larger than 5,000,000 bp for clone 5.

Appendix Figure S5: Validation of MNase digestion patterns before and after size selection (Related to Figure 6 and EV4)

A-B. Chromatin was extracted from two clonal cell lines for each construct or combination (*β^A-globin*, *β-actin*, *β^A-globin*+*β-actin*, 1+2+3, 1+3 and *loxP_LE* (1)+ *β^A-globin*+*β-actin* (3) and partially digested with exponentially increasing concentrations of micrococcal nuclease (MNAse; 2.5, 10, 40 and 160 U/mL).

A. After purification, DNA molecules were subjected to a size selection process that removed most DNA molecules over 1000 bp.

B. The four digested DNA samples obtained for each clonal cell line were subjected to electrophoresis in a 1% w/v agarose gel before and after size selection and stained with SYBR safe. The DNA size marker was a commercial 1 kb plus ladder.

Appendix Figure S6: Validation of the semi-quantitative PCR approach used to determine the spatial proximity of different combination of *cis*-regulatory elements separated by 30 kb (Related to Figure 5)

A-B. Images of agarose gels used for the quantification of specific products obtainted after either the large 1+3 and *loxP_LE* (1)+ β^A*-globin*+*β-actin* (3) excision in 1+2+3, 1+3 and *loxP_LE* (1)+ β^A*-globin*+*β-actin* (3) clonal cell lines or the large *β-actin* (1+3) excision in *β-actin* (1+3) clonal cell line are shown. After 1, 8, 24 and 48 h of 4-hydroxytamoxifen treatment, genomic DNA was extracted and quantified by semi-quantitative PCR. PCR products specific for the large 1+3 or *loxP_LE* (1)+ β^A*-globin*+*β-actin* (3) excision (3.4 kb, in a thick black lines, Up and Low large excision, A left gels), and for the large *β-actin* (1+3) excision (542 bp, in a thick black lines, Up and Low large excision, B left gels) or used for normalization (1.9 kb, amplification from both chromosomes, in a thick black line, Ctrl, A and B right gels) were run on a 0.8% or 1% w/v agarose gel, respectively, and stained with SYBR safe. The DNA size marker was a commercial 1 kb plus ladder.

Appendix Figure S7: PCR validation of clones selected for homologous recombination

A-H. Schematic diagrams showing genomic region containing a site-specific integrated construct. The 5′ and 3′ arms of the targeted vector are shown as black boxes. Arrows #1 and #2 represent primer sets used for the analysis of correct integration of the constructs by homologous recombination. Other arrows represent primer sets used for the analysis of the correct excision of the *BsR* gene at site 2 (#3 and #4) or the correct excision of the *PuroR* gene at site 3 (#5 and #6). PCR products were subjected to electrophoresis in a 1-1.5% w/v agarose gel and stained with SYBR safe. The DNA size marker was a commercial 1 kb plus DNA ladder (M). Lanes marked with a red star correspond to clonal cell lines selected for further analysis.

A-C. Insertion site 1, containing the *β-actin* construct, one *loxP_LE* element or the β^A*-globin*+*β-actin* construct is shown.

D. Insertion site 2 containing the *GFP* reporter construct is shown.

E-F. Insertion site 3, containing the β^A*-globin*+*β-actin* or the *β-actin* construct construct is shown.

G-H. The two genomic regions containing the β^A*-globin*+*β-actin* construct inserted at the late 1 or late 2 site are shown.

A-H. The sizes of the different PCR products obtained after amplification with primers #1 and #2, to check for correct integration, are 2.4 kb (A), 2.6 kb (B), 2.3 kb (C), 2.6 kb (D, left gel), 2.3 kb (E except the last right gel), 2.9kb (F), 2.6 kb (G) and 2.2 kb (H). The sizes of the different PCR products for the screening of cell lines correctly recombined for the *BsR* gene cassette after amplification with primers #3 and #4 are 2.1 kb (D, insertion site 2, right gel) or 1.2 kb with the primers #5 and #6 (F, insertion site 3, last right gel). The clonal cell line containing the *GFP* reporter construct without the *BsR* cassette at site 2 (D, 1-*BsR*) was used for further insertions at site 1 (B, #2 and C, #3 to #6) or at site 3 (E, #1 to #4). The clonal cell lines containing the *GFP* reporter construct without the *BsR* cassette at site 2 and one autonomous replicon at site 1 (C, #3, #4, #5) were used for further insertions at site 3 (E #5 to #9). The clonal cell lines containing the *GFP* reporter construct without the *BsR* cassette at site 2 and one *loxP_LE* at site 1 (B, #2) were used for further insertions of an autonomous replicon at site 3 (E, #10 to #12). Clone 1 with the β^A-*globin* construct inserted at site 1, as previously obtained (Hassan-Zadeh 2012, Fig.6B), was used for the further insertion of a β^A-*globin*+*β-actin* construct at site 3 (E #13 to #18). Clonal cell lines containing two minimal β^A-*globin* modules at sites 1 and 3 were obtained after excision of the *PuroR* cassette at site 3 (E, -*PuroR*, #13 to 18). Clone 2 with the *β-actin* construct inserted at site 1 (B, #2) was used for further insertions of the *β-actin* construct at site 3 (F, #19 to #23).

Appendix Figure S8: Validation of transgene integration into the same chromosome

A. The insertion of transgenes into the same or a different chromosome was analyzed by long-range PCR, with a primer set composed of an upstream primer binding within one construct and a downstream primer binding to the other construct. The amplicons generated after PCR amplification to test for insertion into the same chromosome are represented by red arrows (LR1+2, LR2+3, LR1+3, LR loxP_LE (1)+ β^A-*globin*+*β-actin* (3), LR β^A-*globin* (1+3), LR *β-actin* (1+3)). The absence of an amplicon indicates that the two constructs were inserted into distinct chromosomes. The quality of the DNA was checked with primer sets amplifying, on the two chromosomes, a genomic region located between the two insertion sites (black arrows, LR 1+2 ctrl, LR 2+3 ctrl, LR 1+3 ctrl).

B. PCR products were subjected to electrophoresis in a 0.8% w/v agarose gel and stained with SYBR safe. The DNA size marker used was a commercial 20 kb plus ladder. 1+2 clonal lines #3 and #4 were used to generate 1+3 clonal lines #1 and #2 with the *GFP* reporter construct inserted at site 2 on the same chromosome and 1+2 clonal line #2 was used to generate 1+3 clonal lines #3 to #5 with the *GFP* reporter construct inserted at site 2 on the other chromosome. Clonal line with the *GFP* reporter construct inserted at site 2 was used to generate one clonal line with the *loxP_LE* element inserted at site 1, which itself generated *loxP_LE* (1)+ β^A*-globin*+*β-actin* (3) clonal lines #1, #2 and #3 with the *GFP* reporter construct inserted at site 2 on the other chromosome. Clonal line 1 with the β^A*-globin* construct inserted at site 1, as previously obtained (Hassan-Zadeh 2012, Fig.6B), was used to generate β^A*-globin* (1+3) clonal lines #1 to #6. Clonal line with the *β-actin* construct inserted at site 1 was used to generate *β-actin* (1+3) clonal lines #1 to #5.

Appendix Figure S9: Additional filtering of Hi-C interactions

A. Heatmap of interaction frequencies for p-arm of chr1, demonstrating the position of the insertion site (red arrow) relative to filtered translocations (blue arrows).

B. Raw and unfiltered interactions profiles for allele 1+3 and allele 2 with chr1 p-arm, observed in two clones and their combination, with the corresponding EV1 compartment track. Translocations are highlighted on the compartment track with light blue color and with blue arrows. The insertion site is highlighted with a red arrow.

C-D. Raw and unfiltered interactions profiles for allele 1+3 and allele 2 with chr1 and chr9, observed in two clones and their combination. Blue arrow indicates the location of the β^A*-globin* promoter.

Table 1: Summary of all clonal cell lines (previously published or new clonal lines) analysed in this study

Table 2: Transgene copy number determination in clonal cell lines

The table shows the qPCR results obtained with genomic DNA extracted from the clones selected for the experiments. For each clone, 2 ng of genomic DNA was amplified with a primer set amplifying a sequence within the construct (With) and another primer set amplifying a sequence 5 kb downstream from the insertion site for both alleles (Both). The ratio of the amounts of DNA obtained with the With and Both primer sets was used to determine transgene copy number in all clonal cell lines. For each line, the construct for which the copy number was tested is indicated with the corresponding insertion site.

Table 3: Primers sets used for plasmids constructions

Table 4: Primer sets used for quantitative PCR

Table 5: Characterization of the reference sequences of allele 1+3/2

Characterization of the sequences of allele 1+3 and allele 2 inserts and raw interactions involving the inserts obtained from Hi-C experiments for clones 4 and 5. Characterization of the entire chr1 is added as a reference.

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