Supplementary Methods

Fluorescence *in situ* hybridization

Semi-confluent cells in a 15-cm dish were collected 6 hours after treatment with 100 ng/mL colcemid (Sigma). The samples were fixed, denatured, and spread as described previously (Kondratova et al., 2015; Marotta et al., 2013; Watanabe et al., 2011). To analyze Colo320DM cells, a BAC clone, RP11-440N18 containing *MYC*, and a plasmid containing a part of the 8.5-kb region were prepared using BACMAX DNA Purification Kit (Epicentre) and FastPlasmid Mini kits (5PRIME), respectively. The DNA was labeled with Biotin- or DIG-Nick Translation Mix (Roche) and hybridized onto the metaphase samples at 37ºC for two days with one μg human CotI DNA (Life Technologies) and ten μg salmon sperm DNA. The biotin- or digoxigenin-labeled DNA probe was visualized by Alexa488-conjugated streptavidin (Life Technologies, S-11223) and biotinylated anti-streptavidin antibody (Vector, BA-0500) or by anti-digoxigenin-Rhodamine, Fab fragments (Roche, 11207750910), respectively. The samples were counterstained with ProLong Gold Antifade Reagent with DAPI (Life technologies). Fluorescent images were obtained using Leica DM IRB fluorescence microscope, Leica PL Fluotar L 63x, Leica N Plan 100x Oil objective lens, and Optronics camera system model S99802.

To analyze mouse T3 cells, two BAC clones, RP23-442F1 for cMyc and RP23-322C4 for Pvt1 were labeled with Cy3- and FITC-dUTP by nick translation. The samples were counterstained with ProLong Gold Antifade Reagent with DAPI (Life technologies). Fluorescent images were obtained using Leica DM IRB fluorescence microscope, Leica PL Fluotar L 63x, Leica N Plan 100x Oil objective lens, and Optronics camera system model S99802.

RNA analyses

RNA Extraction: Total RNA was extracted from cells using the RNeasy Plus Kit (Qiagen) and the extracted RNA was treated with DNase I (Life Technologies).

Northern Blot: Twenty-five µg of total RNA was loaded onto a 0.9% agarose-formaldehyde gel and separated for 60 min at 100 V. RNA quality and loading intensity was assessed by the integrity and expression of 28S and 18S RNA. The gel was transferred to a positively charged nylon membrane (Amersham Biosciences) for three hr at 75–80 mmHg pressure using the PosiBlot 30–30 pressure blotter and pressure control station (Stratagene). The RNA was UV-crosslinked to the nylon membrane using the Stratalinker 1800 UV crosslinker (Stratagene). The membrane was probed using Probe C. This probe was PCR amplified and cleaned using the Gel Extraction Kit (Qiagen). Primers for Probe C is listed in Supplementary Table S2. The probe was labeled with [α-^{32}P]dATP. The membrane was hybridized overnight at 65°C in modified Church Buffer (0.5 M sodium phosphate, pH 7.2, 7% SDS, 10 mM EDTA) and exposed to Kodak BioMax MS film (Kodak).

RT-PCR: 500 ng of DNase-treated RNA was reverse transcribed with random hexamers using the Superscript First-Strand Synthesis kit (Life Technologies) according to the manufacturer's conditions. The synthesized cDNA (25 ng/µL final) from Colo320DM was amplified using GoTaq DNA polymerase (Promega). The PCR conditions were as follows: 95ºC for 2 min; 30 cycles of 95ºC for 30 s, 60ºC for 30 s, 72ºC for 30 s; and 72ºC for 5 min; reaction volume, 50 µL. Primer are listed in Table S2. PCR results were analyzed on a 1% TBE agarose gel.

Strand Specific RT-qPCR: 1 µg of DNase-treated RNA was reverse transcribed with either a sense or anti-sense primer using the Superscript First-Strand Synthesis kit (Life Technologies) according to the manufacturer's conditions. The synthesized cDNA (10 ng/µL final) was

amplified using iTaq Universal SYBR Green Supermix (BioRad). The PCR conditions were as follows: 95ºC for 10 min; 40 cycles of 95ºC for 30 s, 60ºC for 30 s, 72ºC for 30 s; reaction volume, 13 µL. Cumulative fluorescence was measured at the end of each of the 40 cycles on LightCycler 480 Real-Time PCR System (Roche). All reactions were performed in triplicate. Primers are listed in Table S2.

Western blotting

Western blotting was performed with NuPAGE protein electrophoresis system and Novex WesternBreeze Immunodetection Kits (Life Technologies) according to the manufacturer's instructions. Cells (3×10^6) were washed twice with 0.15M NaCl, fixed with 10% trichloroacetic acid for 30 min on ice, and lysed with 0.3 mL of LDS Sample Buffer (Life Technologies). Primary antibodies to RNase H1 (1:1000; GeneTex, GTX117624) and DNA Polymerase η (1:100; Santa Cruz, sc-17770) were diluted with blocking solution included in the kit.

Polη knockdown

siRNAs targeting *POLH* (Santa Cruz, sc-36289; Ambion Silencer Select, s10791, and s10792) were transfected using the Neon Transfection System (Life Technologies) according to the manufacturer's instructions. Cells (4×10^5) were electroporated with 100pmol of siRNAs (50nM final) with a single pulse of 1700 V for 20 ms. Scrambled siRNAs (Santa Cruz, sc-37007; Ambion Silencer Cy3-labeled negative control No. 1, AM4621) were employed as a control.

Quantitative-PCR was performed on a CFX384 Real-Time PCR Detection System (Bio-Rad), using SsoFast EvaGreen Supermix (Bio-Rad, 45 cycles of 98 ºC for 10 sec and 60 ºC for 10 sec) according to the manufacturer's instructions. All reactions were performed in quadruplicate. Data analysis was done using Bio-Rad CFX Manager Version 3.1. Primers on the DMs for copy number analysis and reference primers on chromosome 17 for a relative copy number analysis are listed in Table S2. DNA from cells transfected with scrambled siRNA was serially diluted to obtain standard curves and relative DNA amount in DNA from cells transfected with si*POLH* was determined. The values from DM-primers were normalized by those from reference primers.Pulse field gel electrophoresis

DNA (from 3x 10^5 cells) was prepared and digested in gel blocks as described previously (Kondratova et al., 2015; Watanabe and Horiuchi, 2005). The samples were separated with the CHEF Mapper XA system (Bio-Rad) in 1% agarose gels with 0.5x TBE buffer using the auto algorithm mode for either 0.3-2.5 Mb (Fig. S1C) or 4-50 kb (Fig. 4A) range. The junctions of the 8.5-kb region were analyzed by Southern hybridization with the probe labeled with [α-^{32}P]dATP.

Overexpression of RNase H1 in Colo320DM cells

Human RNase H1 was amplified from cDNA isolated from Colo320DM cells using a high-fidelity PCR enzyme, PrimeStar MAX (Clontech), and cloned into pcDNA3.1 (Life Technologies) by In-Fusion cloning (Clontech). Primers are listed in Supplementary Table S2. The resulting expression vector (4 μg) was linearized with *Bgl*II and transfected into Colo320DM cells (2 x 10^6) using Neon Transfection System according to the manufacturer's

protocol with one pulse of 1500 V and 20 ms. After 48 hrs of recovery, the cells were selected with 1.2 mg/mL of G418 for two weeks.

References

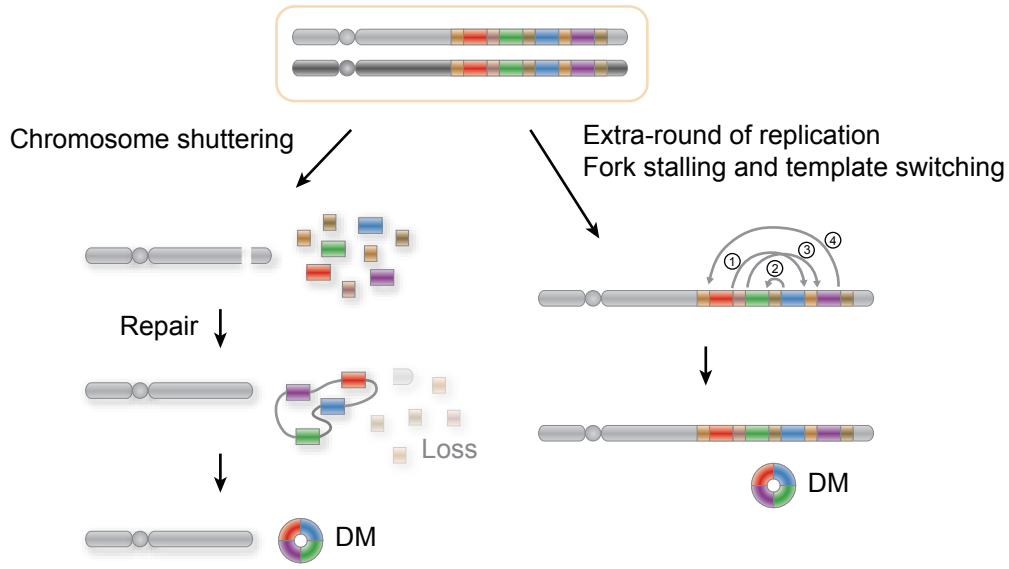
Kondratova, A., Watanabe, T., Marotta, M., Cannon, M., Segall, A.M., Serre, D., and Tanaka, H. (2015). Replication fork integrity and intra-S phase checkpoint suppress gene amplification. Nucleic Acids Res *43*, 2678-2690.

Marotta, M., Chen, X., Watanabe, T., Faber, P.W., Diede, S.J., Tapscott, S., Tubbs, R., Kondratova, A., Stephens, R., and Tanaka, H. (2013). Homology-mediated end-capping as a primary step of sister chromatid fusion in the breakage-fusion-bridge cycles. Nucleic Acids Res *41*, 9732-9740.

Watanabe, T., and Horiuchi, T. (2005). A novel gene amplification system in yeast based on double rolling-circle replication. EMBO J *24*, 190-198.

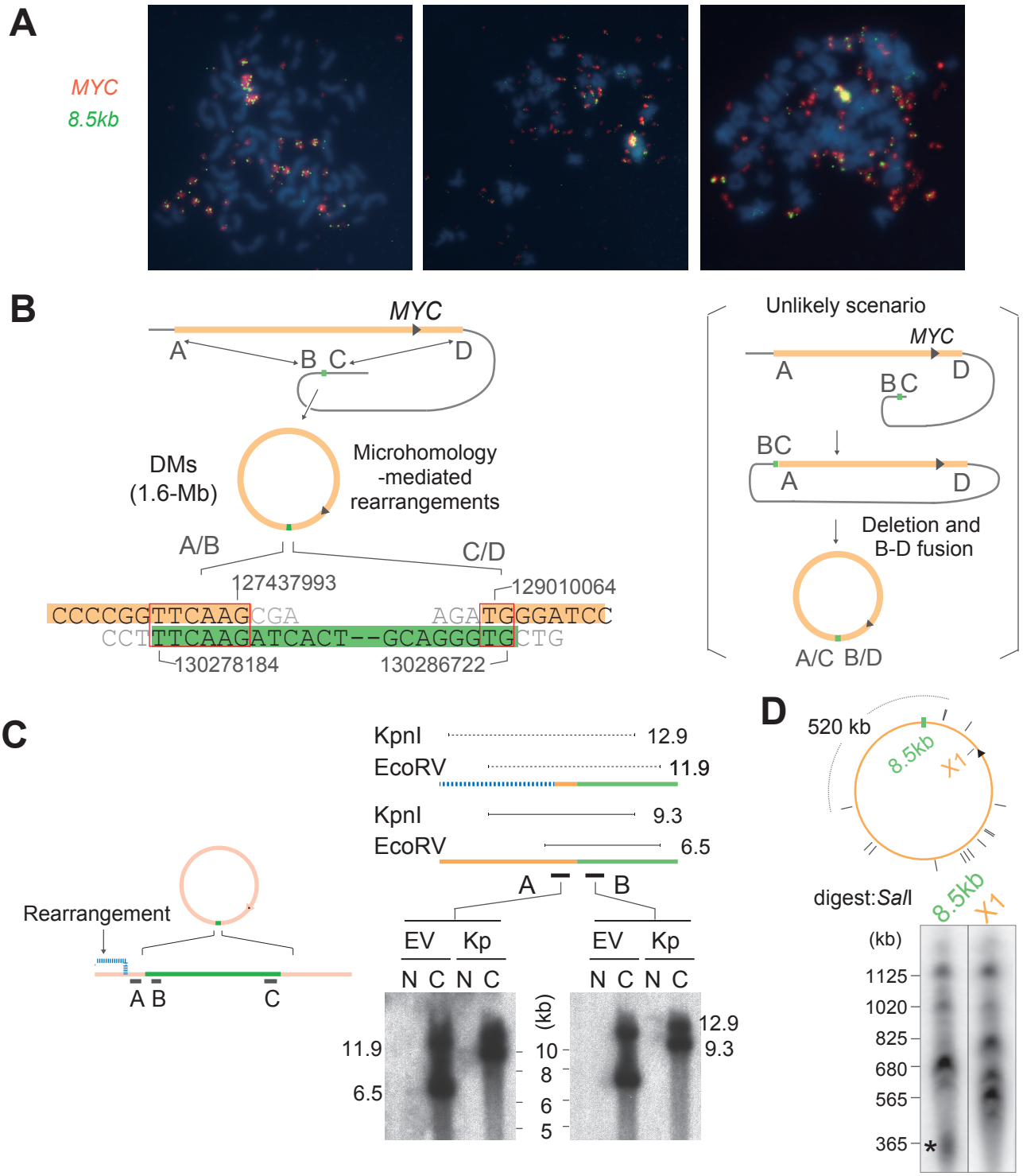
Watanabe, T., Tanabe, H., and Horiuchi, T. (2011). Gene amplification system based on double rolling-circle replication as a model for oncogene-type amplification. Nucleic Acids Res *39*, e106.

Figure S1 - Watanabe *et al*. (related to Introduction)



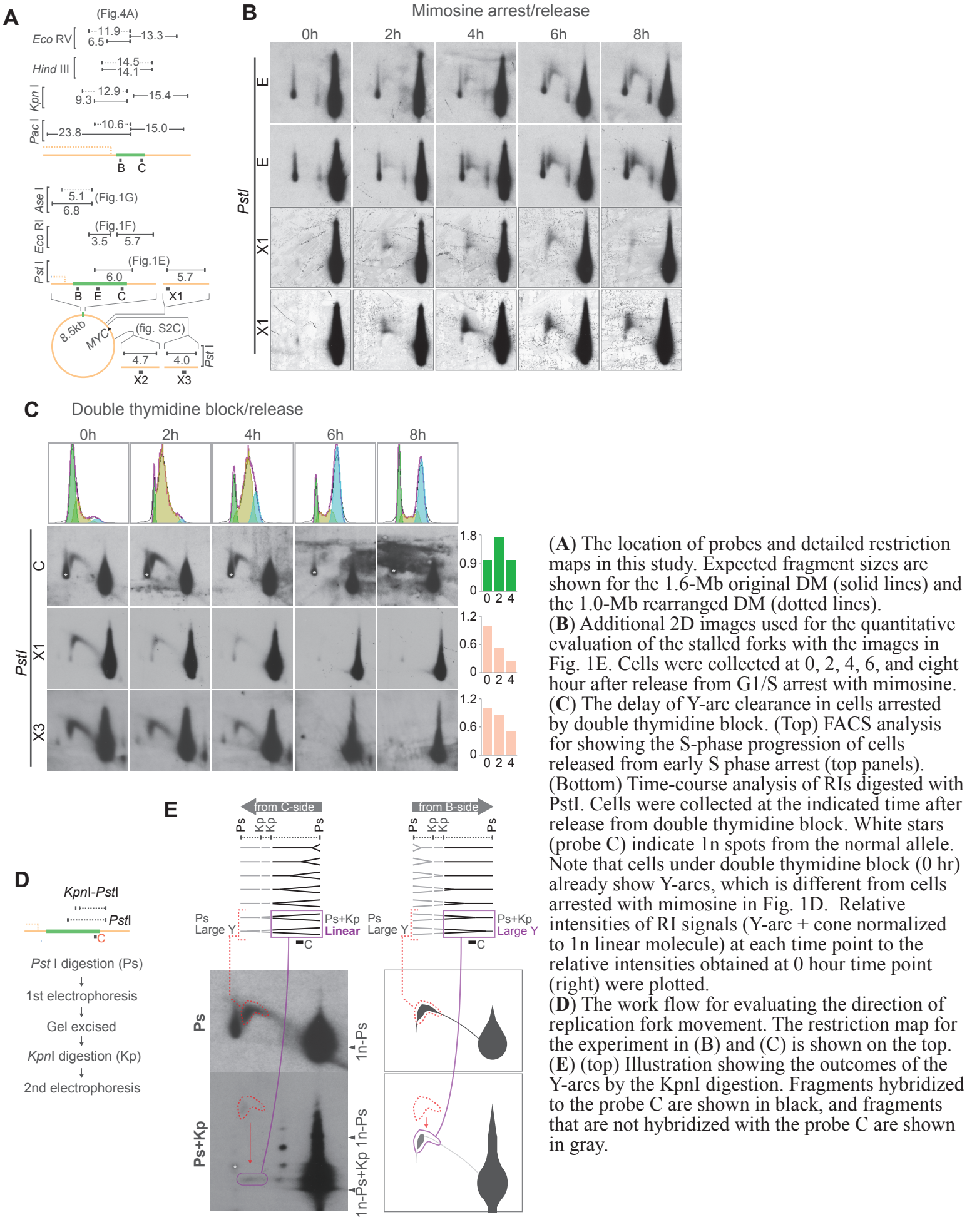
Two mechanisms that integrate multiple genomic segments for the formation of DMs: chromosome shuttering and re-assemble (chromosome breaks and repair) mechanism (left) and replication fork stalling and template switching (FoSTeS) mechanism (right). Two homologues (white and gray chromosomes) are shown at the top. DM formation occurs from the white chromosome.

Figure S2 - Watanabe et al. (related to Figure 1)



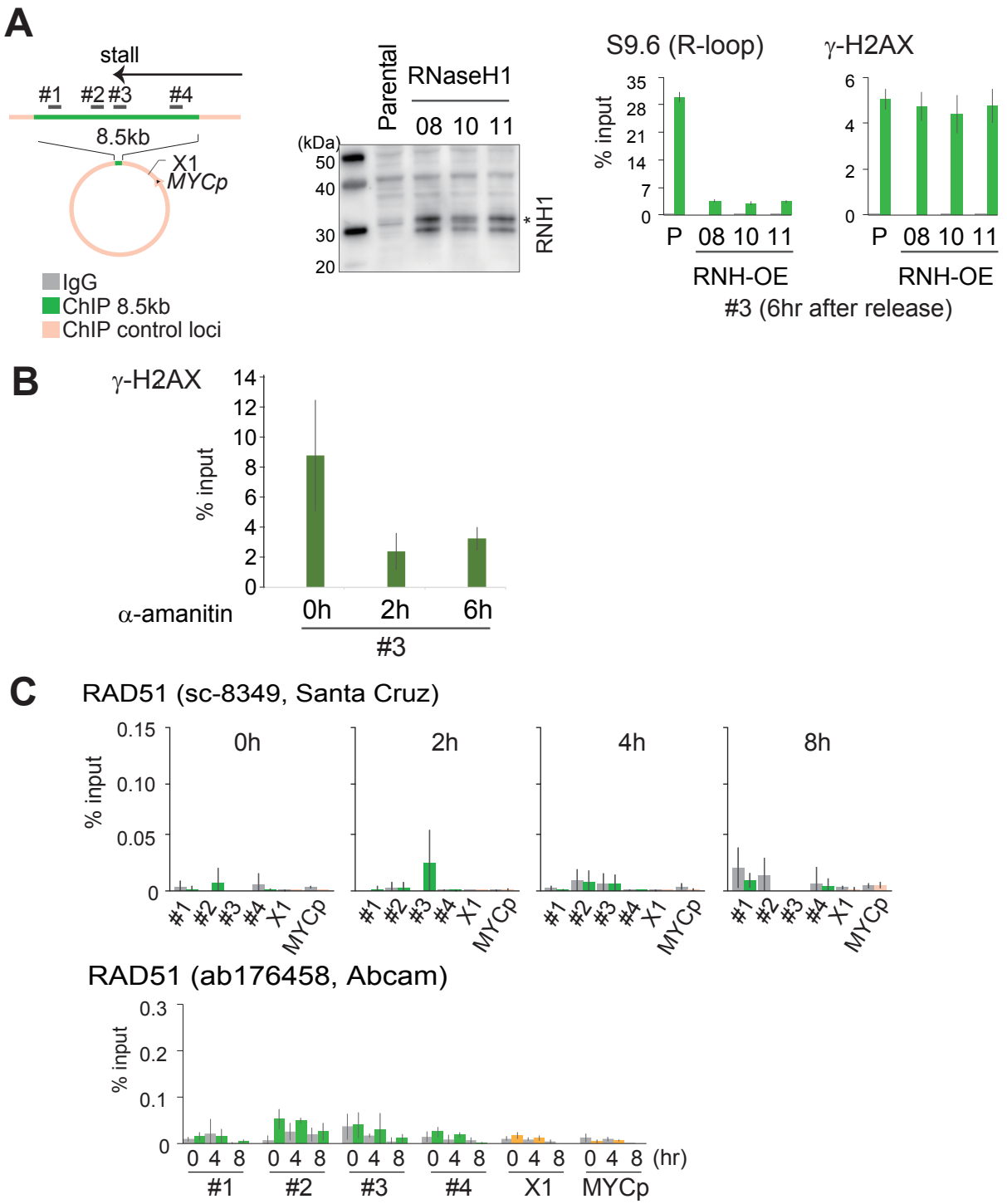
(**A**) Additional Metaphase FISH pictures showing the co-localization between MYC genomic region (red) and the 8.5 kb segment (green). (**B**) (left) A diagram showing microhomology-mediated fusions for the formation of DMs. The 8.5-kb segment is indicated in green. Sequence alignments of the two fusion points are shown with co-ordinates. Red boxes represent microhomologies. (right) The circularization of large chromosomal region harboring MYC would create A-C fusion, and following deletion between the amplified region and the 8.5 kb segment would create B-D fusion. (**C**) A secondary rearrangement (a broken blue line) near the A-B fusion point (left). (right) Southern blot analysis of the fusion junctions. Probes are designed for both sides of the centromeric fusion point (A and B). Restriction enzymes and maps are shown on the top. The probe A and B share two highly amplified restriction fragments. EV, EcoRV; Kp, KpnI. N, normal fibroblast IMR90 DNA; C, Colo320DM DNA. (**D**) Pulse field gel electrophoresis-Southern analysis showing secondary rearrangements within the DMs. (top) the restriction map with the locations of probes 8.5 kb and X1. Radially-oriented gray lines indicate SalI sites (CCCGGG). A predicted fragment hybridizing with the 8.5-kb region is indicated (520 kb). (bottom) PFGE-Southern analysis of SalI-digested genomic DNA.

Figure S3 - Watanabe *et al*. (related to Figure 1)



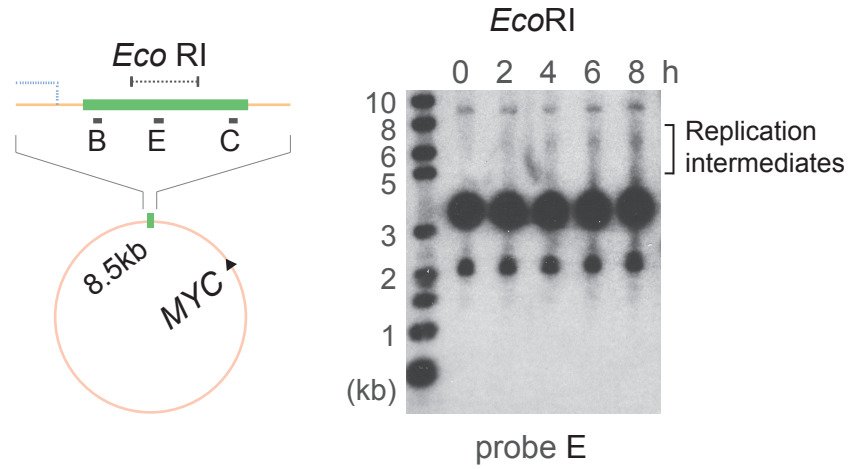
(**A**) The location of probes and detailed restriction maps in this study. Expected fragment sizes are shown for the 1.6-Mb original DM (solid lines) and the 1.0-Mb rearranged DM (dotted lines).
(**B**) Additional 2D images used for the quantitative evaluation of the stalled forks with the images in Fig. 1E. Cells were collected at 0, 2, 4, 6, and eight hour after release from G1/S arrest with mimosine.
(**C**) The delay of Y-arc clearance in cells arrested by double thymidine block. (Top) FACS analysis for showing the S-phase progression of cells released from early S phase arrest (top panels). (Bottom) Time-course analysis of RIs digested with PstI. Cells were collected at the indicated time after release from double thymidine block. White stars (probe C) indicate 1n spots from the normal allele. Note that cells under double thymidine block (0 hr) already show Y-arcs, which is different from cells arrested with mimosine in Fig. 1D. Relative intensities of RI signals (Y-arc + cone normalized to 1n linear molecule) at each time point to the relative intensities obtained at 0 hour time point (right) were plotted.
(**D**) The work flow for evaluating the direction of replication fork movement. The restriction map for the experiment in (B) and (C) is shown on the top.
(**E**) (top) Illustration showing the outcomes of the Y-arcs by the KpnI digestion. Fragments hybridized to the probe C are shown in black, and fragments that are not hybridized with the probe C are shown in gray.

Figure S4 - Watanabe *et al*. (related to Figures 2 and 3)



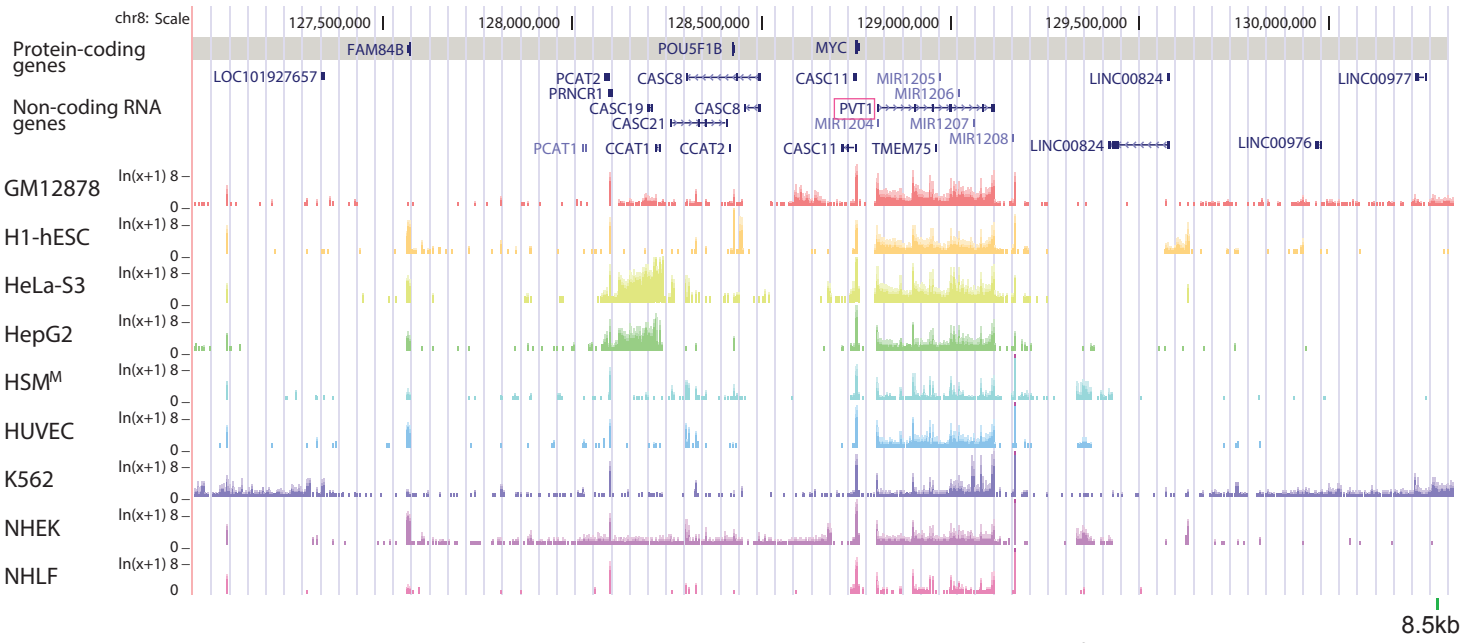
(**A**) Stalled and collapsed forks in Colo320DM cells overexpressing RNaseH1. (left) The location of #3 for evaluating ChIP signals. (middle) Western blot showing the over-expression of RNaseH1 in three independent clones (#08, 10 and 11). (right) ChIP signals for R-loops (s9.6 antibody) and γH2AX in RNaseH1 overexpressing clones. Error bars indicate standard deviations.
(**B**) Inhibition of transcription rescues stalled replication forks. Results of -H2AX ChIP (average of % input from two independent experiments) in untreated cells, cells treated with α-amanitin (250ng/ml) for 2 hours and cells treated with α-amanitin for 6 hours are shown.
(**C**) RAD51 recruitment to the stalled forks in the 8.5-kb segment was evaluated extensively by ChIP-qPCR. (top) RAD51 enrichments were evaluated by ChIP for cells collected at 0, 2, 4, 8 hours after the release from mimosine block. The diagram showing the signals in cells 6 hours after release is shown in the Fig. 3B. Santa Cruz sc-8349 antibody was used. (bottom) ChIP with another RAD51 antibody (Abcam ab176458). Cells were collected at 0, 4 and 8 hrs after release from mimosine block.

Figure S5 - Watanabe *et al*. (related to Figure 4)



Southern analysis of the EcoRI-digested DNA with probe E. Cells were collected at 0, 2, 4, 6 and 8 hours after release from mimosine arrest, embedded in agarose block for DNA extraction and EcoRI digestion and analyzed by low-concentration agarose gel electrophoresis. Note that the accumulation of replication intermediates is evident (black bracket), but no de novo fragments are seen during time course. Left, the location of probes and restriction map (8.5-kb segment) for the analysis.

Figure S6 - Watanabe *et al*. (related to Discussion and Figure 2)



Transcription Levels Assayed by RNA-seq on 9 Cell Lines from ENCODE

Transcription Levels of the 3.5 Mb MYC locus assayed by RNA-seq on nine cell lines from ENCODE. Transcription level for each cell line is shown in a unique color. Note that non-coding RNA gene *PVT1* is highly transcribed in all cell lines.

Table S1. Summary of Segments by Breakpoint (related to Figure 4)

Junction	Segments							
Rearranged 1.0-Mb DM in Colo320DM (human, hg38)								
	129265938	126425748-126426733	126947499-126947514	127547488-127547501	127003434-127003457	127003455-127003874	N.C.	129274476
Mouse T3 cell line (mouse, mm10)								
2-4 junction	61559045	61600176-61600185	61931876-61931886	63051796				
5-7 junction	63249213	59059449-59059306	61968583-61969532	61558957-61559037	62419057-62419067	63616948		

N.C.: Not completed

The genomic locations of sequenced segments were determined using Blat tool (UCSC). The locations of very small segments were determined by aligning the small segments and entire DM sequence using BL2SEQ program (NCBI) and Lasergene software (DNASTAR).

Table S2. List of primers (related to Figures 1-5 and Figures S2-S5)

Target	Location (hg19)		Strand	Sequence (5' to 3')	Remark
qPCR					
chr17-norm.	chr17: 39122731-39122754	hg19	+	AGATTCCAGTTCAGCATTCTCTCG	DNA copy number analysis
	chr17: 39122909-39122930	hg19	-	AACCCACTCCTCGCTTTTGTCG	
chr17	chr17: 39441619-39441643	hg19	+	ACACCACATACTTCTTCCTTCCTCC	DNA copy number analysis
	chr17: 39441781-39441803	hg19	-	GGAAACACAGGCTGGTTTTTCTG	
MYC	chr8: 128748852-128748875	hg19	+	CGGGTAGTGGAAAACCAGGTAAGC	DNA copy number analysis
	chr8: 128748947 128748970	hg19	-	GCAGGAATGGGAGAAAAGACACC	
8.5-kb	chr8: 130282618 130282640	hg19	+	TTAGTGATTTAGGACTGTGGGCG	DNA copy number analysis
	chr8: 130282696 130282719	hg19	-	GGTATGAAAGTAGGAGGCAGGATG	
B	chr8: 130279605-130279628	hg19	+	TCCTCCAACAGGAACAAGTTTGAC	Strand specific RT-qPCR
	chr8: 130279722-130279743	hg19	-	TCTGGCTGTCTTGATGGTTTGC	
C	chr8: 130285601-130285623	hg19	+	CATGTATGGGGTTCTGTGCTACG	Strand specific RT-qPCR
	chr8: 130285810 130285833	hg19	-	CCAGCAGCCTCAAACATTTGTTAC	
8.5-kb, #1	chr8: 130280478-130280502	hg19	+	CTAGCAAGATAAATACGGACTGCAT	ChIP-qPCR
	chr8: 130280603-130280627	hg19	-	ATTTTACTTCAACCCTGAAATAGTG	
8.5-kb, #2	chr8: 130281502-130281526	hg19	+	AATGTCCAACTTTAGTCAACACTGC	ChIP-qPCR
	chr8: 130281697-130281721	hg19	-	AAAGTTGTTCAGCTCCATTAGTCAT	
8.5-kb, #3	chr8: 130282292-130282316	hg19	+	GAGTGATATCCTTGTTCGTAGCTGT	ChIP-qPCR
	chr8: 130282532-130282556	hg19	-	TCTGATATAGGTCCATTCGAGGTTC	
8.5-kb, #4	chr8: 130285031-130285055	hg19	+	AAAATAGCCTTTCCTCTACCAAGTG	ChIP-qPCR
	chr8: 130285166-130285190	hg19	-	TAAATCAAGGACCATCTATCCTAGC	
X1	chr8: 128784874-128784895	hg19	+	GCCTCTCTCAATCCACACCTTG	ChIP-qPCR
	chr8: 128784956-128784979	hg19	-	CTCTGTTCTCTTCTGGGAAACTGG	
MYC promoter	chr8: 128748856-128748881	hg19	+	TAGTGGAAAACCAGGTAAGCACCGAA	ChIP-qPCR
	chr8: 128748965-128748991	hg19	-	TCTGAGAAAAGTGTCAATAGCGCAGGA	
Probes					
A	chr8: 127438189-127438210	hg19	+	CTGCTCTCTTTTTCCAGGGACC	
	chr8: 127438717-127438740	hg19	-	AGCCATTGTCTTGTTGCCTCTAAG	
B	chr8: 130279432-130279455	hg19	+	GCTTGGATGTGGATACAATGCTCC	
	chr8: 130279844-130279864	hg19	-	TCCTCTTTGGCTTACCCTTGC	
C	chr8: 130285421 130285441	hg19	+	ACGGCGTTATGTTTACGGAGC	
	chr8: 130285879 130285902	hg19	-	ACTGGTCATTGAGTTAGGGTTTGG	
D	chr8: 129007865-129007884	hg19	+	AGTGTTTGCGGTGATGGTCG	
	chr8: 129008338-129008360	hg19	-	GAAAGGAGAAAGGTGAGTGTGGG	
E	chr8: 130282618-130282640	hg19	+	TTAGTGATTTAGGACTGTGGGCG	
	chr8: 130283077-130283101	hg19	-	GGAAGGATTCTCAGAGGAACACGAC	
X1	chr8: 128784577-128784600	hg19	+	AAACCAGAGATTCCCCAGGCAAGT	
	chr8: 128785323-128785347	hg19	-	AAGTTCCAATTACAGTTATCCCCTA	
X2	chr8: 128690027-128690051	hg19	+	GGCACATGCTTACAATAATAACAGT	
	chr8: 128690535-128690558	hg19	-	CAAGCAATGACTTTACTTTCCGAA	
X3	chr8: 128768123-128768145	hg19	+	CTTACTTAACTGGAATGACCCTA	
	chr8: 128768629-128768652	hg19	-	AGTTTATTTGGGTTTGATACTTCG	
FISH8.5kb	chr8: 130279930-130279953	hg19	+	GCCTCAATCTTACCACCTTTAACG	
	chr8: 130284932-130284953	hg19	-	CAGAGTTCTGTTCTCCCCGTGG	
DM-copy number analysis in POLH knockdown cells					
X2	chr8: 127678093-127678115	hg38	+	ATAAAGGACATGGTCTGCTTCCC	DM copy number analysis
	chr8: 127678189-127678209	hg38	-	AACCTACGATGAATGCAGCCC	
chr17	chr17: 40416998-40417022	hg38	+	GCAATCTGTTAGTAGGCAGTGGTGG	Reference for DM copy number analysis
	chr17: 40417173-40417195	hg38	-	GCAGGAACTGAAAGAAGTGGGTG	
Cloning or sequencing					
	chr8: 127438895-127438916	hg19	-	TGTTTAGGAGGGGTTTACGGGC	Sequencing for the 1.6-Mb original DM
	chr8: 130279440-130279463	hg19	-	TTTGAGAGGGAGCATTGTATCCAC	Sequencing for the 1.6-Mb original DM
	chr8: 130285881-130285904	hg19	+	AAACCCTAACTCAATGACCAGTGG	Sequencing for the 1.6-Mb original DM
	chr8: 129009794-129009817	hg19	+	TTTTACCTGTAGCCCCTCTGCTGC	Sequencing for the 1.6-Mb original DM
	chr8: 127438695-127438714	hg19	+	ACACACCAAGGGTGTCCTCA	Cloning for the 1.0-Mb rearranged DM
	chr8: 128016101-128016120	hg19	-	GTAATTGCCCTTGCCCCTTG	Cloning for the 1.0-Mb rearranged DM
	chr8: 127439380-127439402	hg19	+	GGCAGAGTTGTGTGGAAGAGGGA	Sequencing for the 1.0-Mb rearranged DM
	chr8: 128016066-128016088	hg19	+	AACCTCCAGTGCCCCTGCTTGCA	Sequencing for the 1.0-Mb rearranged DM
	chr8: 129003827-129003849	hg19	+	TGGGACCTCAGTCATGTGGCAGA	Sequencing for the 1.0-Mb rearranged DM
	chr8: 129009737-129009759	hg19	+	GTAAGATAGTCACCGTGTGGAGC	Sequencing for the 1.0-Mb rearranged DM
	chr8: 127438377-127438399	hg19	+	ACAATGTACAATCTCTAATGTTG	Sequencing for the 1.0-Mb rearranged DM
	chr15: 61558557-61558581	mm10	+	AAATCAGCCTAAAGACAAGTCACAC	Cloning for the T3 cell line
	chr15: 61559070-61559095	mm10	-	CATTCCTTTCTTCATTTCAGGACCTC	Cloning and sequencing for the T3 cell line
	chr15: 63051661-63051686	mm10	+	CGTCAATCTCCCTAAAGTATTCCAGT	Cloning for the T3 cell line
	chr15: 63052229-63052254	mm10	-	ATTCCTGCTGATTTCACTTATATTGT	Cloning and sequencing for the T3 cell line
	chr15: 63248804-63248830	mm10	+	TTGGAAAGAGATGGCATTTGTTCATCA	Cloning and sequencing for the T3 cell line
	chr15: 63249360-63249386	mm10	-	CTCTTGCCTTAATTTCAGATTCATCCC	Cloning for the T3 cell line
	chr15: 63616584-63616609	mm10	+	GTGGTCATTTTGAGAAGCTGAATGTC	Cloning and sequencing for the T3 cell line
	chr15: 63617195-63617221	mm10	-	CTTATACTGACTTCCATCACTACCCTC	Cloning for the T3 cell line
Cloning or sequencing					
	chr2: 3558230-3558260	hg38	-	ATGAGCTGGCTTCTGTTCCTGGCCCACAGAG	Cloning of human RNase H1
	chr2: 3545785-3545814	hg38	+	TCAGTCTTCCGATTGTTTAGCTCCTTCTCT	Cloning of human RNase H1
				CAATCGGAAGACTGAGAATTCCACCACACTGGACTA	pcDNA3.1
				CAGAAGCCAGCTCATGGTGAATTCTGCAGATATCCAGC	pcDNA3.1