

## SUPPLEMENTARY FIGURE LEGENDS

**Supplementary Figure 1. Origin characterization and RNase H-sensitivity of eyebrow arcs.** Panels 1-3; chick mtDNA was digested with *Sall*, *BpmI* or *MscI*, respectively, and fragments separated by 2D-AGE. Panel 4, a map of chick mtDNA marked with the predicted restriction sites of *Sall*, *BpmI* and *MscI* and the relevant probe, c9. Sites in bold are those furthest ‘downstream’ of the NCR (major non-coding region) for each of the enzymes; thus, if effectively unidirectional replication initiates within the NCR (see Fig. 1.13) then the existence of eyebrow arcs (e) must indicate that restriction sites are blocked at multiple positions on one branch of replicating molecules, including sites 13.3 kb (panel 1), 14 kb (panel 2) and 15.2 kb (panel 3) downstream of the NCR. See illustrations beneath panels 1-3 (red crosses – blocked sites, arrows – direction of replication). Panels 7-10: illustrative sets of digests showing that eyebrow arcs (e) are RNase H sensitive. Mouse mtDNA was digested with *BglII* (panels 7 and 8) or *XhoI* (panels 9 and 10), and treated with (panels 8 and 10) or without RNase H (RH) (panels 7 and 9), prior to 2D-AGE and hybridization to probe m7. Belanger et al. (1996) have previously studied duplex and single-stranded eyebrow arcs: the start and end points of the eyebrow were similar. However, the single-stranded eyebrow arc was essentially horizontal (uniform mobility in the second dimension, irrespective of mass). RNase H treatment of the mtDNA eyebrow, which is presumed to create partially single stranded circular molecules by removing much of the nascent L-strand (see discussion), produced an inverted eyebrow (panels 8 and 10), unlike the flattened eyebrow observed by Belanger et al., (1996). The increased mobility of replicating circles of mtDNA

compared to bacterial plasmid DNA may reflect the fact that the former consists of a growing duplex branch attached to a partially single-stranded circle, whereas the latter comprises a single-strand branch and a fully duplex circle. The shape of mtDNA eyebrow arcs following RNase H treatment can also be attributed to partial conversion of L-strand RNA to DNA concurrent with leading strand DNA synthesis in a substantial fraction of molecules (see discussion).

**Supplementary Figure 2. Origin mapping and RNase H-sensitivity of slow-moving**

**Y-like arcs.** Panel 1: *Bsa*HI-digested chick liver mtDNA hybridized with probe c2. The simple Y arc is associated with the unit-length (1n) fragment, nt 8,800-15,152. Additionally an fSMY arc is seen, which is attributable to restriction site blockage at nt 15,152 and at 16,645, and which extends the fragment to nt 1,436 (panel 1). This interpretation is supported by the fact that the same arc hybridized to probe c1, designed to detect the linear fragment nt 16,645-1,436 (data not shown). The fSMY arc lies very close to the linear duplex arc; hence, the unidirectional origin (or replication terminus) must be located very close either to nt 16,645 or to nt 1,436. However, the latter is ruled out by the results of the double digest *Msc*I/*Bcl*II, (see Fig. 3.1-3.4 of the main paper), since the associated fSMY arcs in this case started and ended well above the linear duplex diagonal. Panel 2 is identical to Fig. 2.8, and is shown for reference alongside panel 3, which is an identical digest treated with RNase H (RH). Panels 4 and 5: *Bcl*II-digested mouse liver mtDNA, either untreated (panel 4) or treated with RNase H (panel 5), and hybridized with probe m4. Note that fSMY arcs are highly sensitive to RNase H in both species. Panel 6: *Eco*RV-digested mouse mtDNA, hybridized to probe m3. The

simple Y spans nt 14,829-5,369, with an accompanying fSMY arc resulting from restriction site blockage at nt 14,829. Note the relative signal strengths of the fSMY and simple Y arcs in panels 4 and 6, indicating that a majority of replicating mtDNA molecules in mouse liver must have incorporated RNA at the sites indicated. Relevant *EcoRV*, *MscI* and *BsaHI* sites are indicated on the circular maps in Figs 1 and 2, respectively. A representative intermediate from each fSMY arc is illustrated (not to scale) immediately below the relevant 2D gel image, gray box – NCR, O<sub>H</sub> – ‘origin of heavy strand replication’.

**Supplementary Figure 3. Distinguishing between RITOLS and the strand-displacement model of mtDNA replication.** A combination of one nascent DNA strand and one nascent RNA strand predicts widespread restriction site blockage and RIs that are duplex on all branches. In contrast, the orthodox strand-displacement model (Robberson et al., 1972) (Brown et al., 2005) predicts partially single stranded RIs and failure to cleave a restriction site only if the endonuclease is unable to cut its recognition site on single-stranded DNA. *HhaI* cuts its recognition site in either single-stranded or double-stranded DNA and hence provides a diagnostic test to distinguish the two models of replication. As shown in the main paper, *HhaI* was unable to cleave fSMY arcs of chick mtDNA (Figs. 3.13-3.19). The pattern of mtDNA RIs was identical when mtDNA was incubated with *HhaI* for periods ranging from 15 min and 2 h (data not shown) suggesting that sites did not remain uncut merely because single-stranded DNA is cleaved less efficiently than duplex DNA. Likewise *AccI*, another enzyme capable of cleaving single-stranded DNA, yielded an fSMY arc with mouse mtDNA (panel 1 and

interpreted in panels 2 and 3). RNase H-sensitivity indicates, furthermore, that fSMY arcs are composed largely of RNA-DNA hybrid on at least one branch. The apparent molecular weights of the various species in the first dimension of electrophoresis, which resolves according to molecular weight, correspond closely with those of the predicted duplexes. This is illustrated for mouse and chick liver mtDNAs digested with enzymes shown (which do not cut ssDNA), and hybridized, respectively, with probe m1 (panel 4) and c4 (panel 5), alongside dsDNA size markers as indicated. The predicted, apparent molecular weights of fully duplex and partially single-stranded fSMY arcs expected under the two replication models are shown beneath the 2D-AGE gel panels. There are no data available on the precise paths of partially single-stranded arcs predicted by the strand-displacement model. However, it is possible to deduce some aspects of their behaviour. It is well established that duplex and single-strand DNA molecules produce distinct arcs on 2D-AGE. Therefore, it is highly implausible that fSMY arcs of partially single-stranded DNA of diverse size would consistently coincide with arcs of fully duplex intermediates. If against all probability fSMY arcs of the strand-displacement model did mimic duplex fSMY arcs on 2D-AGE then there is no rational explanation for the changes mediated by RNase H (see supplemental Figs. 2 and 4, and Figs. 2, 4 and 5 of the main manuscript).

**Supplementary Figure 4. Distribution of ribonucleotides across the mitochondrial genome in mouse and chick liver.** Panel 1 - interpretation of Fig. 4.7, indicating how components of an fSMY arc can be converted to a simple Y arc. X – blocked restriction site, cyan lines – nascent DNA, red lines – RNA incorporated on lagging strand. Arrows

indicate the direction of fork movement. This interpretation is based on the observation that most of the signal in the fragment (nt 1,634-6,086) is in the simple Y arc, even though *XmnI* does not cut ssDNA or RNA/DNA hybrid (Fig. 4.7), plus the fact that it is largely RNase H-sensitive (Fig. 4.8). Most replicating mtDNA molecules in which the replication fork has passed through nt 6,086 but not yet reached nt 1,634 must therefore be DNA on all four strands at nt 6,086, i.e. are matured at the site soon after the fork has passed beyond it (in mouse liver), but an RNA lagging strand is nevertheless being laid down by the progressing fork (see schematic drawing A in panel 1, and the discussion section). The partial fSMY arc (B in panel 1) represents molecules where the leading strand is advancing towards nt 1,634, and in which maturation at nt 6,086 has yet to occur. Panels 2, 3: the same applies to the *NspI* fragment spanning a closely overlapping region (nt 118 – 6,096), and treated with RNase H (RH) (panel 3), mock RNase H treated (data not shown) or left untreated (panel 2). Panels 4, 5: in contrast, the simple Y arc of *BanII* fragment nt 9,047-12,588 was unaffected by RNase H (interpreted in panels 7 and 8), although the electrophoretic mobility of the RIs visualized as slow-moving arcs A and B (panel 5) was altered by RNase H treatment (A' and B', panel 6), consistent with the loss of a substantial amount of RNA from one branch of these RIs. Panels 9-12: analysis of the corresponding region of chick liver mtDNA suggests that maturation start sites are more widely distributed than in mouse liver and also vary from molecule to molecule. Note that the stem and loop-forming region known as O<sub>L</sub> in mammals lies in a 5 tRNA gene cluster, specifically between the tRNA<sup>Asn</sup> and tRNA<sup>Cys</sup> genes (nt 5,160-5,191); no intergenic region is found at the junction of these two genes in chick mtDNA (nt 6,504). The digest shown in panel 9 (*HpaI*, hybridized with probe c9) is of the equivalent region

to that in mouse liver, shown in Fig. 4.5. Whereas the signal in the latter is mainly found in an SMY arc (arrowed and illustrated), in chick liver much more of the signal is in the simple Y arc, indicating differences in maturation to DNA between chick and mouse, in the region beyond the tRNA<sup>Asn</sup> / tRNA<sup>Cys</sup> gene junction. In the case of the *HincII* digest shown as panel 10, hybridized with probe c9, there must in some replicating molecules be blockage of the restriction site at nt 6,470, but cleavage of the site at nt 7,907, in order to generate SMY arc A, whereas blockage at nt 7,907 has to occur in other replicating molecules in order to generate arc B. Similarly, whilst SMY arc A in panel 11 (*MscI* digest, probed with c10) is compatible with maturation commencing from within the 5 tRNA cluster, arc B indicates that the site at nt 8,334 must remain blocked while the fork advances from nt 4,566 to 1,600, i.e. that no maturation has initiated. In this gel image the unit-length fragment and associated simple Y arc are not seen, since they are of much greater electrophoretic mobility and had exited the gel. Furthermore, in panel 12 (*BsgI* digest, probed with c10), SMY arc A is the result of site blockage at nt 5,249, but cleavage at nt 1,597 and 7,608, whereas SMY arc B is generated by site blockage also at nt 7,608, but cleavage at nt 1,597 and 10,807. No such additional arcs are seen in corresponding fragments of mouse liver mtDNA digested with *XmnI* (Fig. 4.7) or *NspI* (panel 3), consistent with a more uniform pattern of initiation of maturation. Species C in panel 10 is an SMY arc with blocked sites at nt 6,470 and 5,923. In panel 11, e is an eyebrow arc and species C is a fSMY arc with blocked sites at nt 15,947, 8,334 and 4,566. Panel 13, map showing restriction sites of the enzymes applied in panels 9-12, 14, 15. Panels 14 and 15, 2D-AGE analysis of *AflIII* digested chick liver mtDNA treated without and with RNase H, respectively, and probed with c10; note the limited effect of

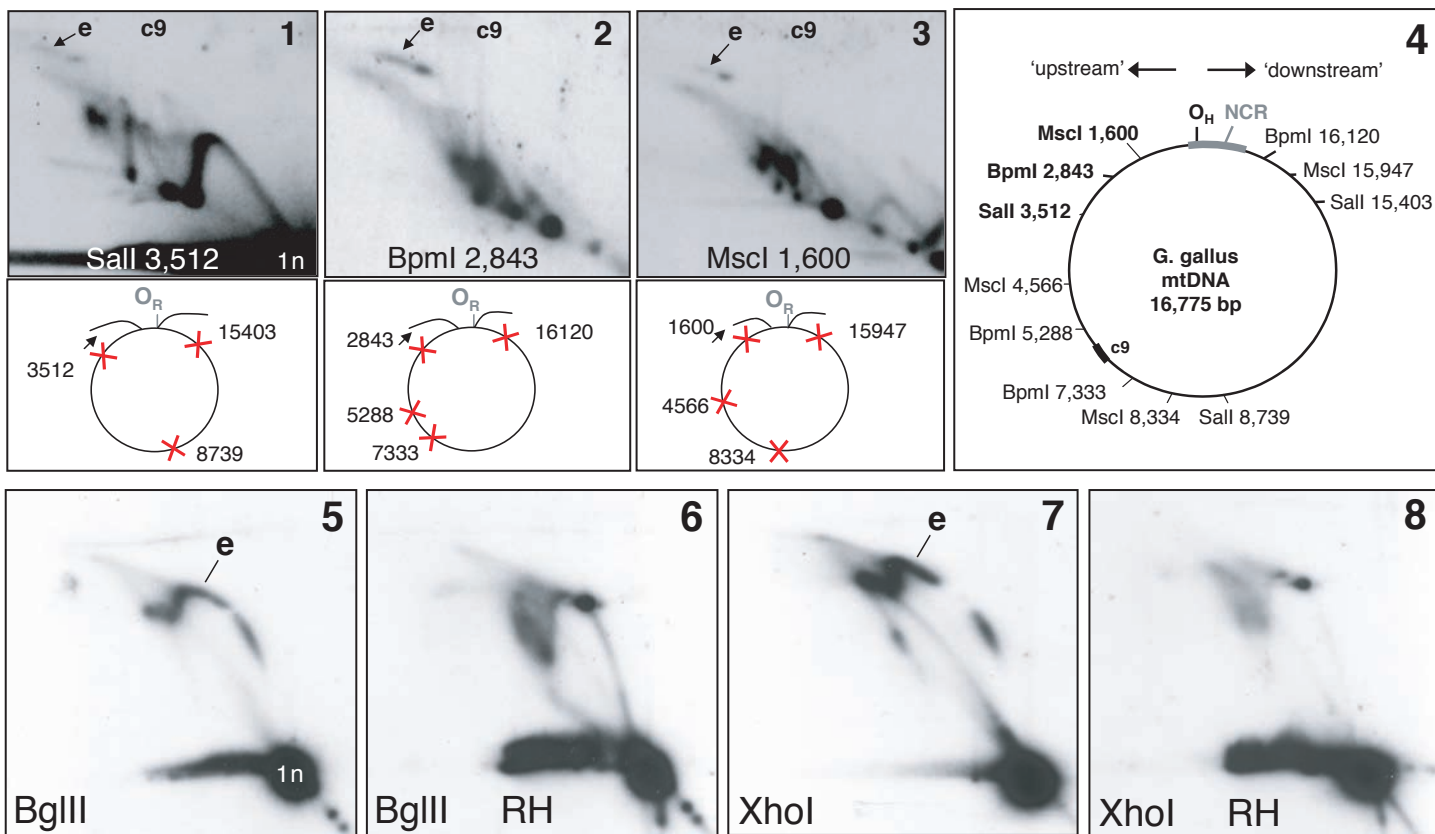
RNase H on the simple Y arc compared to similar fragments of mouse mtDNA (Fig. 4.7, 4.8, and supplemental Fig. 4.2, 4.3) showing that the simple Y arc is less sensitive to RNase H. Cleaving chick mtDNA with *Eco*NI, which has a site within the NCR, yielded a combination of fSMY and SMY arcs, panel 16 (and interpreted in panels 17 and 18), as per mouse mtDNA (Fig. 4.11). Panels 19-21; *Ban*II-digested mouse liver mtDNA hybridized sequentially with probes m8, m3 and m4, respectively. The combination of fSMY and SMY arcs, illustrated below each gel image and interpreted in panel 22, suggests initiation of replication occurs upstream of nt 15,749 in some molecules.

**Supplementary Figure 5. Clubheaded initiation arcs are RNase H sensitive in rat and chick mtDNA.** Rat liver mtDNA, extracted by the same protocol as for mouse liver, was digested with *Btg*I, which cuts twice in the genome (nt 224 and 11,099), and hybridized with probe r1, spanning nt 15,765-16,120 (Gadaleta et al., 1989). A prominent clubheaded bubble arc, b2 (arrowed, panel 1) was detected, extending over most, but not all, of the fragment, which was truncated further by RNase H treatment (RH, panel 2), but not by incubation in RNase H buffer without enzyme (data not shown). Note the persistence, after RNase H treatment, of the residual complete initiation arc, b1. Clubheaded initiation arcs associated with O<sub>H</sub>-containing fragments of chick mtDNA were also sensitive to RNase H (panels 3-6). Restriction enzymes and the boundaries of the O<sub>H</sub>-containing fragments are indicated above each panel.

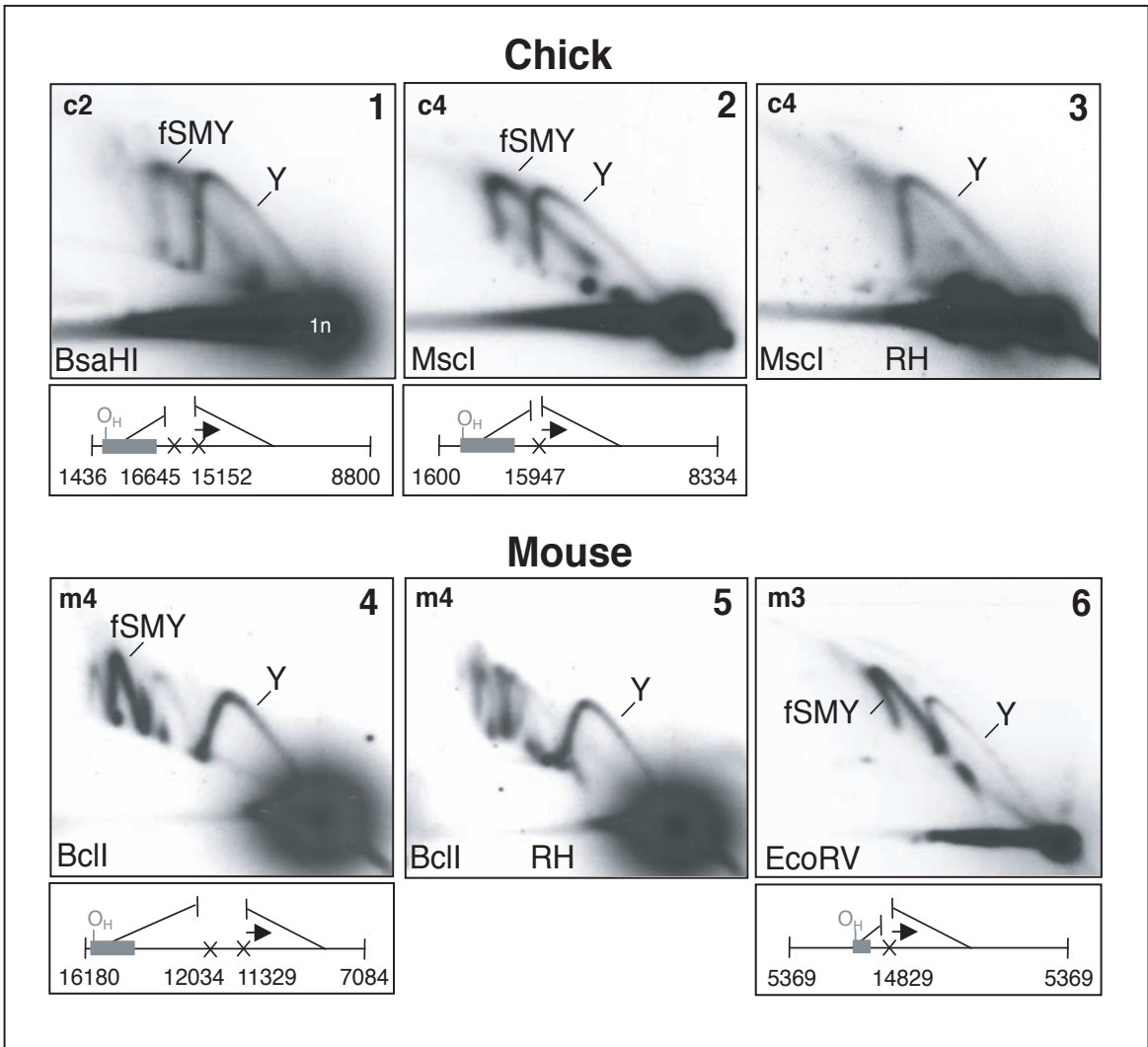
## References

- Belanger, K.G., Mirzayan, C., Kreuzer, H.E., Alberts, B.M. and Kreuzer, K.N. (1996)  
Two-dimensional gel analysis of rolling circle replication in the presence and  
absence of bacteriophage T4 primase. *Nucleic Acids Res*, **24**, 2166-2175.
- Brown, T.A., Cecconi, C., Tkachuk, A.N., Bustamante, C. and Clayton, D.A. (2005)  
Replication of mitochondrial DNA occurs by strand displacement with alternative  
light-strand origins, not via a strand-coupled mechanism. *Genes Dev*, **19**, 2466-  
2476.
- Gadaleta, G., Pepe, G., De Candia, G., Quagliariello, C., Sbisà, E. and Saccone, C. (1989)  
The complete nucleotide sequence of the *Rattus norvegicus* mitochondrial  
genome: cryptic signals revealed by comparative analysis between vertebrates. *J  
Mol Evol*, **28**, 497-516.
- Robberson, D.L., Kasamatsu, H. and Vinograd, J. (1972) Replication of mitochondrial  
DNA. Circular replicative intermediates in mouse L cells. *Proc Natl Acad Sci U S  
A*, **69**, 737-741.

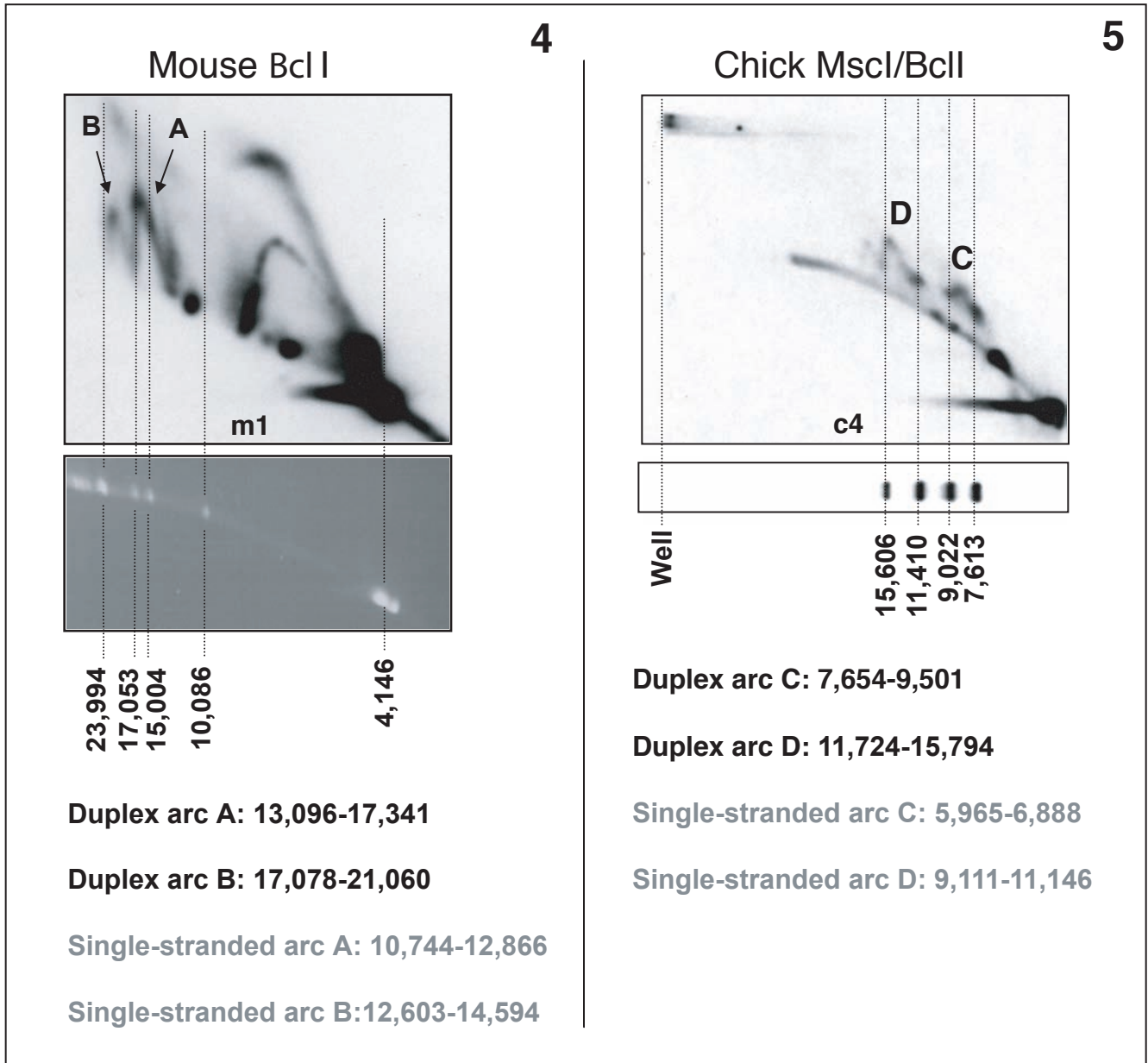
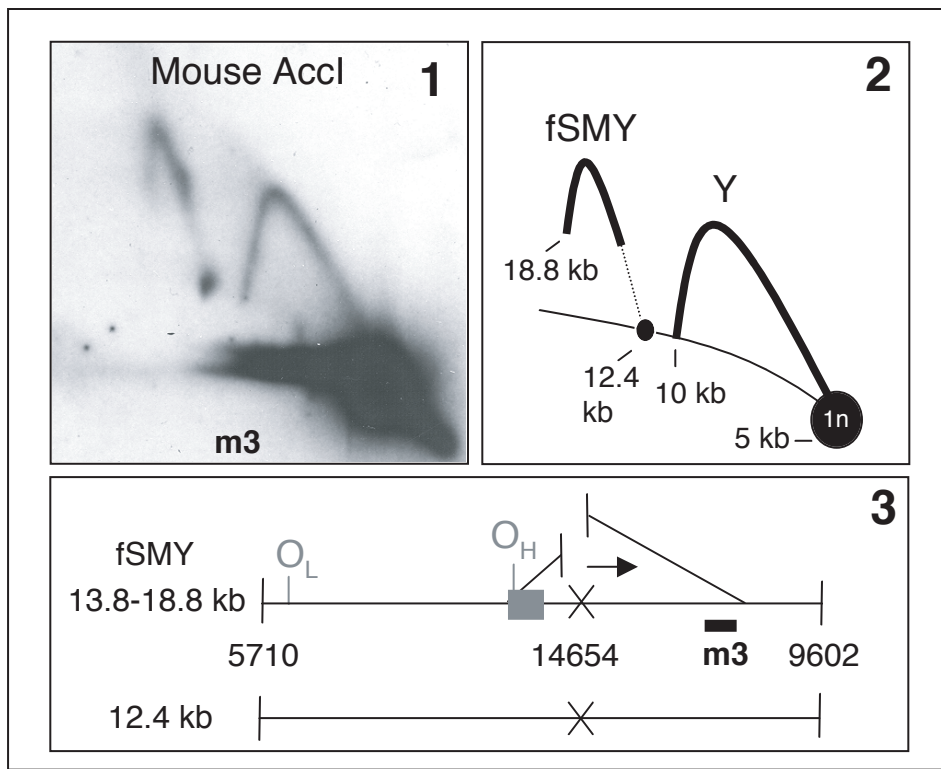




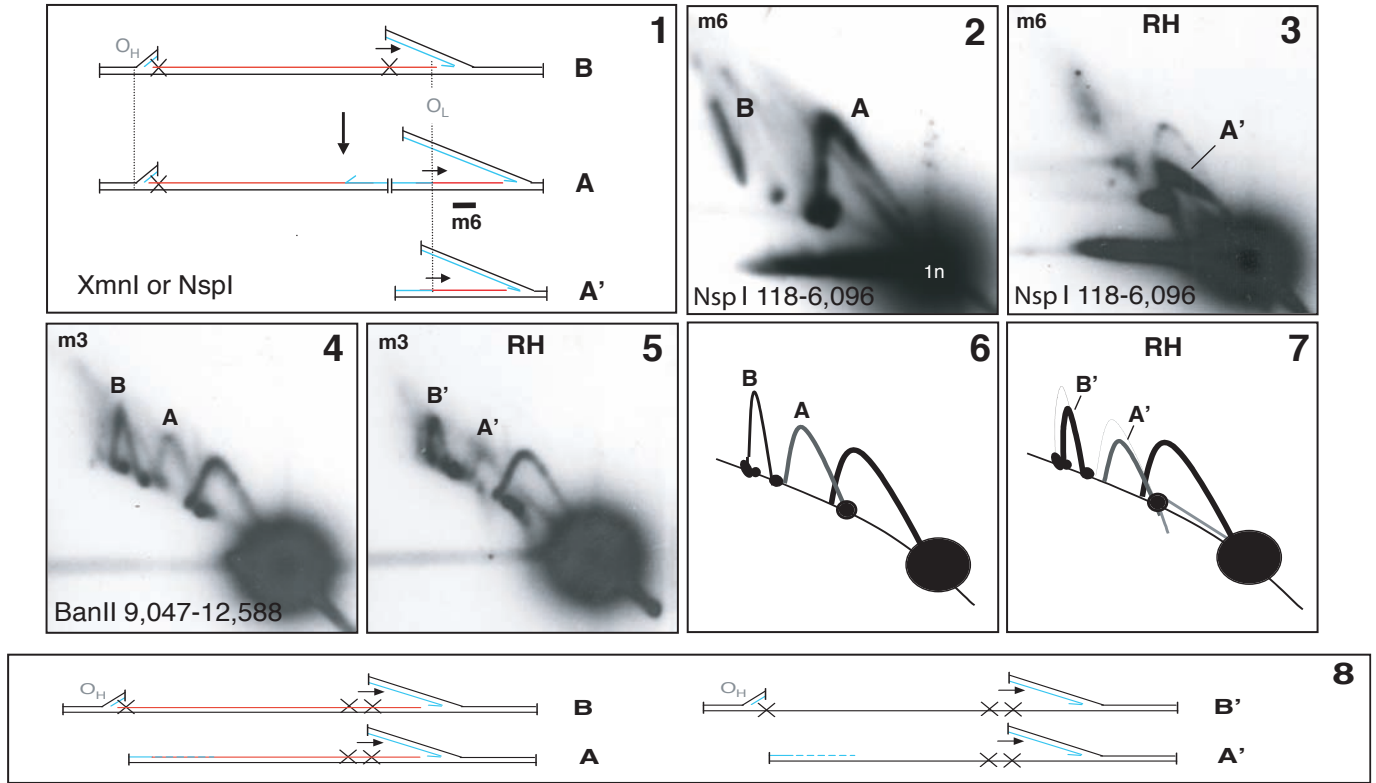
Yasukawa et al., Supplementary Fig. 1



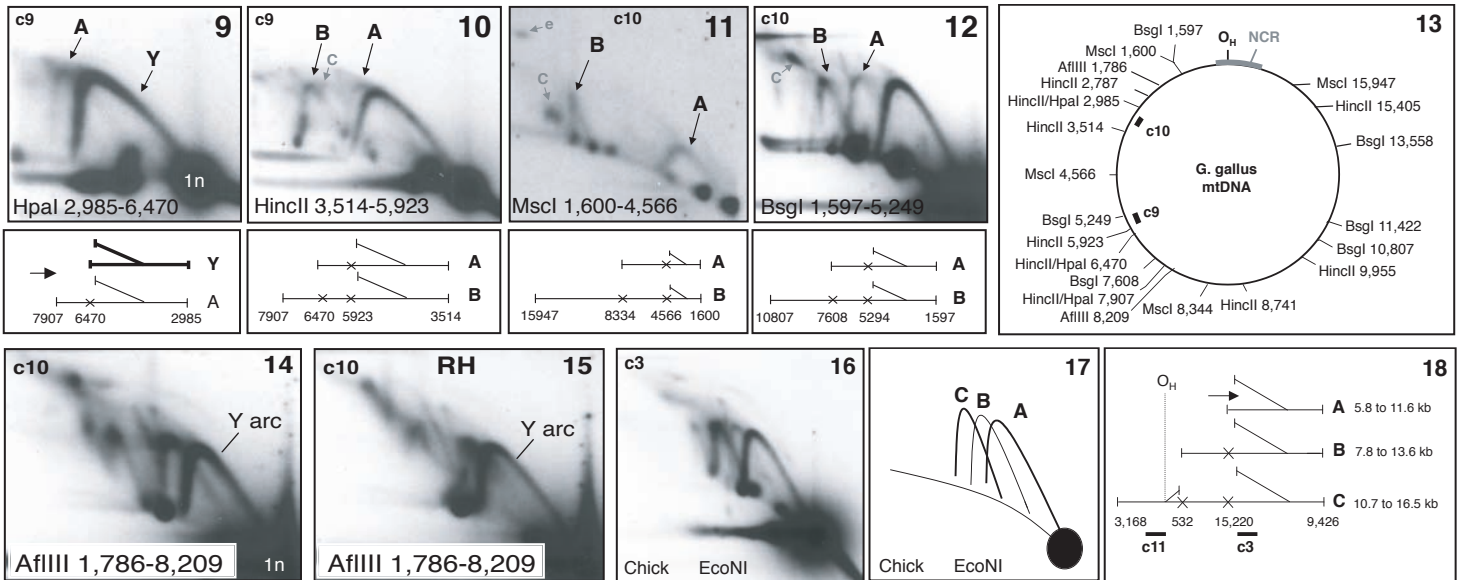
Yasukawa et al., Supplementary Fig. 2



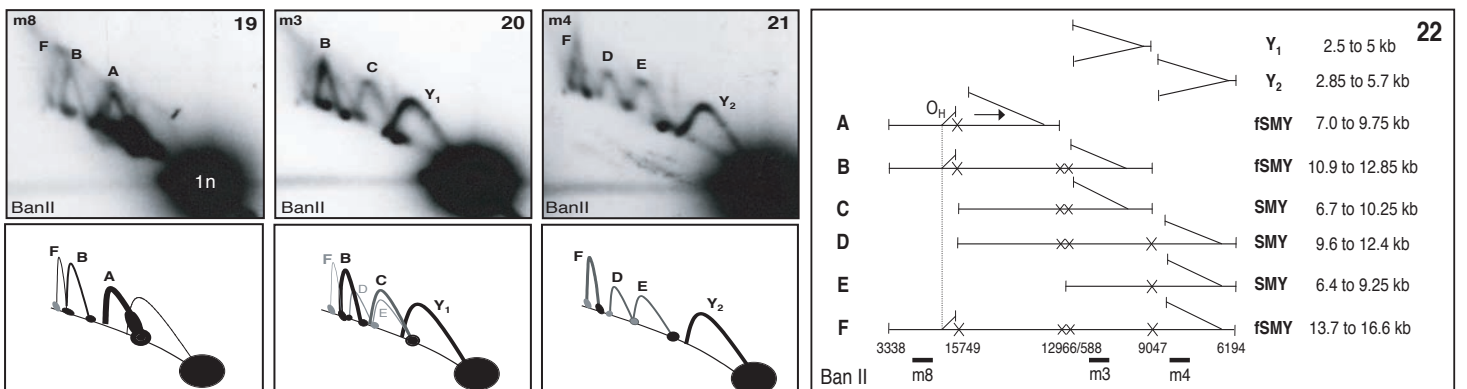
## Mouse

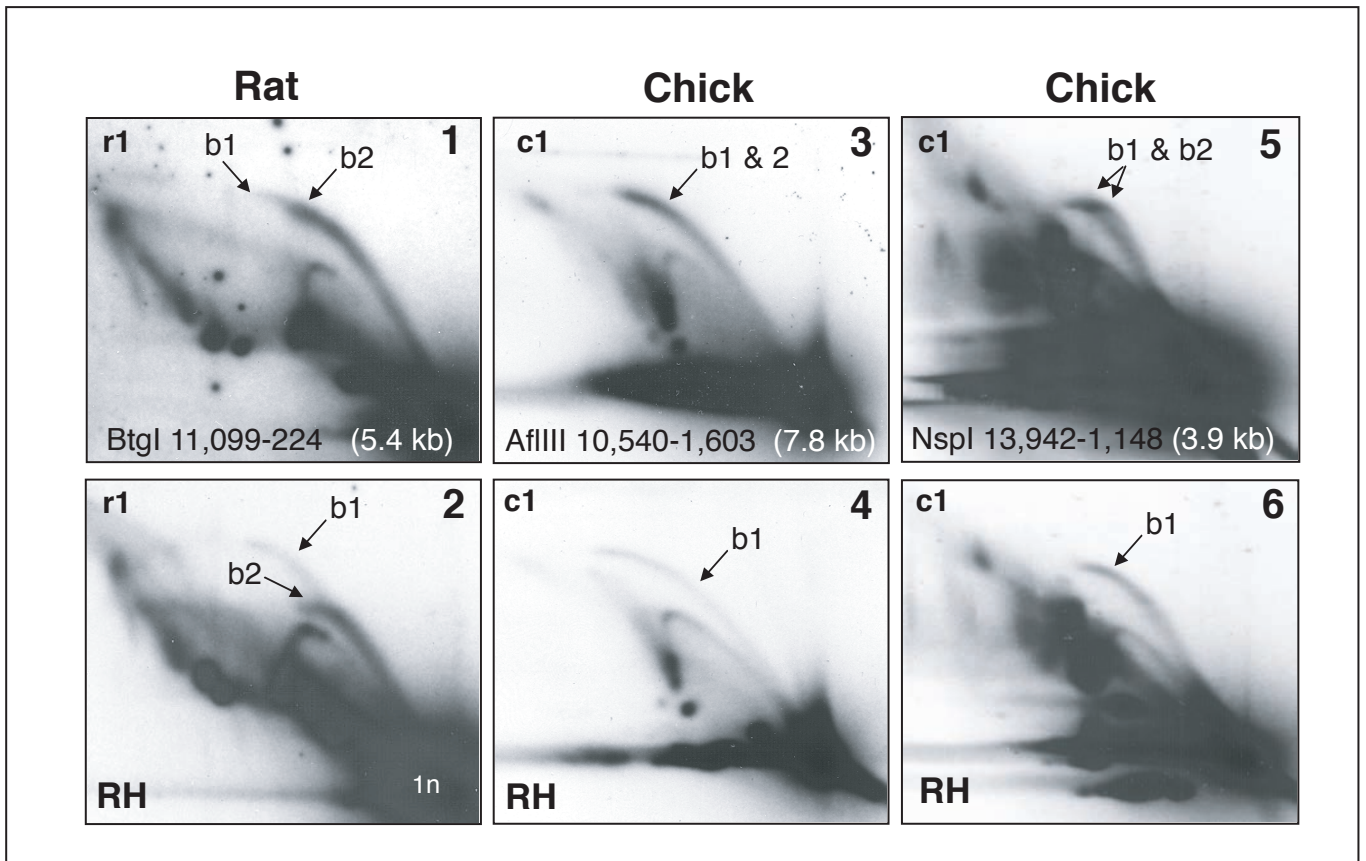


## Chick



## Mouse





Yasukawa et al., Supplementary Fig. 5