1 **Truncation of gene *F5L* partially masks rescue of vaccinia virus strain**

2 **MVA growth on mammalian cells by restricting plaque size**

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23 **Summary**

24 Modified Vaccinia virus Ankara (MVA) is a candidate vaccine vector that is severely attenuated due

25 to mutations acquired during several hundred rounds of serial passage *in vitro*. A previous study

26 used marker rescue to produce a set of MVA recombinants with improved replication on

27 mammalian cells. Here we extend the characterisation of these rescued MVA strains and identify

28 vaccinia virus (VACV) gene *F5L* as a determinant of plaque morphology, but not replication *in vitro*.

29 F5 joins a growing group of VACV proteins that influence plaque formation more strongly than virus

30 replication and which are disrupted in MVA. These defective genes in MVA confound the

31 interpretation of marker rescue experiments designed to map mutations responsible for the

32 attenuation of this important VACV strain.

33

34 **Main text**

35 Modified Vaccinia virus Ankara (MVA) is a leading candidate vector for recombinant poxvirus

36 vaccines (Gomez *et al.*, 2008). MVA was the result of several hundred rounds of serial passage

37 starting with the virulent strain chorioallantois vaccinia virus Ankara (CVA) in primary chicken

38 embryonic fibroblasts (Mayr *et al.*, 1975). In contrast to the broad host range typical of VACV, MVA

39 fails to replicate in all but a few mammalian cell lines (Carroll & Moss, 1997, Drexler et al., 1998,

40 Jordan et al., 2009, Okeke et al., 2006). The full genomic sequences of CVA and MVA have been

41 published (Antoine et al., 1998, Meisinger-Henschel et al., 2007). In addition to six large deletions

42 (termed Deletion I to VI),mutations affect coding in more than 60% of the annotated ORFs of MVA

43 compared with CVA (Meisinger-Henschel et al., 2007, Meyer et al., 1991). However, the mutations

44 responsible for the host range restriction of MVA *in vitro* and its attenuation *in vivo* remain

45 unknown.

46

47 MVA does not form plaques on monolayers of most mammalian cells and this characteristic can be

48 exploited in marker rescue experiments to map genetic lesions underlying the restricted host

49 range. Using cosmids with genomic fragments from a replication-competent VACV strain, referred

50 to as 'Ankara', Wyatt et al (1998) made a set of rescued MVAs that replicate on mammalian cells.

51 These rescued MVAs were selected on the basis of increased plaque size on BS-C-1 cells and the

52 work broadly mapped the location of the mammalian replication defect of MVA to several regions

53 at the left end of the genome (Wyatt *et al.*, 1998). One known host range gene, namely SPI-1,

54 which resides in deletion I, was repaired in some of the rescued MVAs (Shisler *et al.*, 1999). While

55 SPI-1 may contribute to the host range defect, other work has shown that the major deletions

56 (even in combination) cannot account for the replication defect of MVA on mammalian cells in

57 general (Dimier et al., 2011, Meisinger-Henschel et al., 2010).

58

59 We began by following up apparent differences in plaque morphology across this set of rescued

60 MVAs (Melamed et al., 2013, Wyatt et al., 1998). BS-C-1 and HeLa cells were infected with MVA,

61 Ankara or the rescued MVAs and foci or plaques formed under semisolid media (0.4% w/v

62 carboxy-methyl cellulose) were immunostained (Staib *et al.*, 2004) at 72 hours post infection

63 (h.p.i.). MVA failed to form plaques or foci on HeLa cells but small foci made up of a few tightly

64 packed cells were seen on BS-C-1. The rescued MVAs exhibited a range of plaque morphologies

65 on BS-C-1 and HeLa cells. A striking difference was seen between the plaques of v51.2 and v44.1

66 grown on BS-C-1: v51.2 infected cells formed tightly packed piles whereas infection with v44.1

67 caused the formation of obvious plaques with clearance of the monolayer at the centre (Fig 1a).

68 The independently rescued v51.1 and v44.2 lineages also formed piles and plaques respectively

69 (not shown). By contrast, and consistent with the previous report, we observed no difference in

70 replication rates of v51.2 and v44.1 on BS-C-1, HeLa or IEC-6 cells in multiple step growth

71 analyses (Fig 1b-d) (Wyatt *et al.*, 1998).

72

73 The three cosmids used to produce the rescued MVAs (namely c51, c44 and c47) cover the left

74 end of the VACV genome, but recombination sites have not been defined (Fig 2a). Three of the

75 major deletions of MVA (deletions I, V and II) lie within the region shared by c51 and c44 (Meyer *et*

76 *al.*, 1991). A simple PCR-based analysis of the rescued MVAs revealed that deletion I, but not V or

77 II, was repaired both in v51.2 and v44.1 (not shown), consistent with reported PCR detection of the

78 SPI-1 (deletion I) but not K1L (deletion II) host range genes (Wyatt *et al.*, 1998). By contrast, all

79 three deletions were repaired in v44/47.1, v44/47.2, v51.1 and v44.2. The relatively small repairs in

80 v44.1 and v51.2 made these an attractive pair to study further. As an aside, examination of

81   sequences surrounding the deletions suggest that Ankara is not closely related to CVA and MVA,

82   confirming a recent report (Melamed et al., 2013). Cosmids c44 and c51 overlap substantially and

83   both include most of the *Hind*III C fragment and the small *HindIII* N, M and K fragments. However

84   compared with c51, c44 extends further rightwards and into the start of the *HindIII* E fragment

85   (Wyatt *et al.*, 1998). This suggested that gene/s in the *Hind*III F region, unique to c44, were

86   responsible for the larger plaques made by v44.1. To test this, six genes: *K6L, F1L*, *F5L*, *F11L*,

87   *F12L* and *F13L,* distributed across this region were sequenced for v44.1, v51.2 and Ankara and

88   compared with those published for MVA. As expected, sequences from v51.2 matched those of

89   MVA for all six genes but in v44.1, *K6L*, *F1L, F5L*, and *F11L* matched Ankara and so were repaired

90   in this virus (region shown in Fig. 2c).

91

92   To map the gene/s responsible for the plaque phenotype we carried out a set of marker rescue

93   experiments. Firstly, *K6L-F4L* and *F5L-F11L* from Ankara were cloned into plasmids to bisect the

94   region of interest. BHK-21 cells infected with v51.2 (m.o.i.=0.05) were transfected with 1 µg of

95   linearized plasmid using Lipofectamine 2000 (Invitrogen). At 48 h.p.i., virus was harvested and

96   used to infect BS-C-1. A single large plaque was isolated after recombination between the v51.2

97   genome and the *F5L-F11L* plasmid. This virus (v51.2/F5L-F11L) was plaque purified and found to

98   contain repaired versions of two truncated genes, *F5L* and *F11L*. *F5L* is transcribed early and

99   predicted to encode a 36.5kDa major membrane protein (Yang et al., 2010, poxvirus.org). The

100   MVA homologue lacks 104 aa of the c-terminus, including a putative transmembrane domain. *F11L*

101   is required for efficient release of virus particles from infected cells, normal plaque size in vitro and

102   virus spread in vivo (Cordeiro et al., 2009, Morales et al., 2008). Next we tested whether repair of

103   *F5L* or *F11L* alone in v51.2 might produce larger plaques. The transfer plasmids for these

104   experiments included a *GFP/bsd* marker under the control of the VACV strong synthetic promoter

105   downstream of the gene to be repaired (Wong *et al.*, 2011). This allowed visual (eGFP) and drug

106   (blasticidin) selection of recombinant viruses in addition to possible increases in plaque size. A

107   complication of adding *GFP/bsd* downstream of *F5L* and *F11L* is that the promoters of adjacent

108   genes (*F4L* and *F10L*, respectively) are separated from their ORF. For this reason, these promoter

109   sequences were repeated after the *GFP/bsd* marker cassette (Fig. 2d). These direct repeats also

110   make the marker unstable in the absence of drug selection. The *F5L* and *F11L* rescue plasmids

111   were linearised and transfected into BHK-21 cells infected with v51.2 (m.o.i.=0.05). Viruses with

112   plaques larger than v51.2 were isolated after transfection with *F5L* (v51.2/F5LGb) and *F11L*

113   (v51.2/F11LGb) and after 3-4 rounds of plaque purification on BS-C-1, the fidelity of repairs were

114   verified by sequencing. In the case of v51.2/F5LGb, further passage allowed the isolation of a virus

115   that had lost the *GFP/bsd* marker, but retained the repair of *F5L* (v51.2/F5L).

116

117   Having isolated these viruses we compared plaque phenotypes and sizes (Fig 3a, b). Repair of

118   *F5L* alone (v51.2/F5LGb and v51.2/F5L) had a strong effect on plaques: they were larger and there

119   was significant monolayer clearance in the centre. The repair of *F11L* also increased plaque size

120   but did not lead to clearance of cells from their centres. Further, the effect of *F5L* and *F11L* was

121   additive because plaques made by v51.2/F5L-F11L were larger than those of viruses with repairs

122   of *F5L* and *F11L* alone. Next we tested virus growth and found that neither *F5L* (with or without

123   *GFP/bsd*), or *F11L* altered the replication of v51.2 in single or multiple step growth curves (Fig 3d,

124   e). Finally, the use of the *GFP/bsd* marker allowed us to isolate an MVA with *F5L* repaired

125   (MVA/F5LGb). Repair of *F5L* did not improve MVA replication or change plaque size on BS-C-1

126   cells (Fig 3c, f).

127

128   Wyatt et al (1998) concluded that multiple genes must be involved in the host range defect of MVA

129   because non-overlapping cosmids improved replication and additive effects on plaque size were

130   observed when multiple regions were repaired. However, their data are also consistent with a

131   model where more than one gene can rescue replication, but multiple genes contribute to plaque

132   size. We believe this latter model is a better explanation for the profound variation in plaque size

133   but narrow range of virus titres obtained on BS-C-1 for the rescued MVAs as previously reported

134   (Wyatt *et al.*, 1998). It is also supported by the recent finding that v51.1, with a smaller plaque,

135   replicates to higher titres on Vero cells than v44/47.1 (Melamed *et al.*, 2013). From the literature,

136   three VACV proteins that increase plaque size without enhancing replication are inactive or

137   missing in MVA, namely C2, F11 and O1. C2 is a kelch protein that is required for the usual distinct

138   borders of plaques made by VACV strain WR, but is lost from MVA, due to major deletion V (Pires

139   de Miranda et al., 2003). As noted above, F11 plays roles in virus-induced cell motility (Valderrama

140   *et al.*, 2006) and in normal plaque size (Cordeiro et al., 2009, Morales et al., 2008). O1 is required

141   for sustained activation of the RAF/MEK/ERK pathway and is truncated in MVA. Deletion of *O1L*

142   decreases the plaque size of CVA (Schweneker *et al.*, 2012). Despite their association with altered

143   plaques, none of these genes has a strong influence on growth of VACV *in vitro* and for *O1L* and

144   *F11L* this has been shown for MVA (Antoine et al., 1998, Morales et al., 2008, Pires de Miranda et

145   al., 2003, Schweneker et al., 2012). *F5L* is now the fourth VACV gene function missing from MVA

146   that is required for normal plaques, but not replication.

147

148   We determined the status (repaired or not) of each of these four genes and plaque phenotypes

149   across the full set of rescued MVAs allowing some further observations (Fig. 3g). 1) Of all the

150   rescued MVAs, v44/47.1 has the largest plaques but their size remains smaller than Ankara. This

151   suggests that genes outside the region mapped by Wyatt et al (1998) affect plaque size or

152   replication. 2) The similarity (no significant difference in size) between v51.2/F5L-F11L and v44.2

153   suggests that the individual contribution of *C2L* to plaque size is minor. 3) Repair of *F5L* and *F11L*

154   increased plaque size, but *F5L* was required for the clearance of cells from the middle of plaques.

155   Repairing both genes gave an additive increase in plaque size and together these suggest that F5

156   and F11 act independently. 4) Plaques from v44/47.1 were larger again than v51.2/F5L-F11L

157   suggesting that a gene in the region covered by c47 also has a strong influence on plaques. The

158   most likely candidate here is *O1L*, consistent with results obtained when this gene was deleted

159   from CVA (Schweneker *et al.*, 2012). 5) Restoration of F5 and F11 to v51.2 gave larger plaques

160   than v44.1. *F5L* and *F11L* are intact in v44.1, but the repairs in this virus do not extend as far to the

161   left of the genome as in v51.2. It seems likely that this region also contains genes that affect

162   replication or plaque formation (Fig 2a, b).

163

164   In summary, we have identified the truncation of F5 as a determinant of plaque morphology but not

165   *in vitro* replication in MVA. Further, the existance of *F5L* and several other genes required for

166   normal plaque formation complicate the interpretation of work done to map attenuating mutations

167   of MVA, which has assumed plaque size is an accurate surrogate for replication. We also show

168   here that the relatively small single repair in v51.2 alone produces a substantial improvement in

169   replication on three mammalian cell lines. Together these lead us to conclude that the range of key

170   genomic changes associated with the replication defect of MVA in mammalian cells has been

171   previously overestimated.

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Figure legends

Fig 1: Rescued MVAs show a range of plaque size and morphology on BS-C-1 and HeLa

cells. (a) Representative plaques formed by the viruses shown on BS-C-1 or HeLa cells under

semisolid media. Cells were immunostained at 72 h.p.i. (100× final magnification, scale bars

100µm). (b-d) Multiple step growth analysis (m.o.i.=0.01) in (b) HeLa, (c) BS-C-1, or (d) IEC-6.

Cells were incubated for 1h with virus then washed and fresh media added. 0 h.p.i. samples were

harvested immediately after addition of fresh media. Cell associated virus collected at 24, 48 and

72h.p.i. was titrated and divided by virus titre present after absorption. Data points represent mean

± SEM of three independent wells.

Fig 2: MVA genome and repairs in v51.2 and v44.1 (a) Map of the MVA genome showing the

location of the six major deletions, indicated by grey boxes and the approximate location of

cosmids used to make the rescued MVAs (blue bars). *Hind*III fragments of Copenhagen are

marked for reference. (b) Approximate locations of identified repairs in v51.2 and v44.1 are

indicated in relation to the rescuing cosmid (blue bar). Repairs surrounding deletion I in v51.2 and

v44.1 are indicated by the green boxes and a second repair, unique to v44.1, by the red box. (c)

Mapping of the repair unique to v44.1: ORFs shown in black are truncated in MVA compared with

CVA. Genes shown in white are identical between CVA and MVA. Genes shown in grey contain

small mutations in MVA (4 aa deletions in F1L and F3L, single aa substitution in F8L). Genes

labelled in red were sequenced in v44.1 and v51.2 to identify the extent of the repair. For these

four genes v44.1 matches Ankara not v51.2 or MVA.(d) Structure of *F5L* region in v51.2/F5LGb.

F5L is followed by *GFP/bsd* driven by a strong synthetic promoter (not shown) and flanked by

repeated sequences (rpt) to preserve the promoter for *F4L*. The structure of *F11L* in v51.2/F11LGb

was similar.

Fig 3: Restoration of *F5L* or *F11L* to v51.2 alters plaque morphology but not replication. (a-

b) Plaques formed on BS-C-1 cells under semisolid media by the recombinant viruses shown were

immunostained 72 h.p.i. (a) Representative plaques, original magnification 100× (scale bar

278   100µm). (b) Areas of individual plaques are plotted with the average shown by the solid line. (***

279   v51.2 significantly different to all other viruses (p<0.001), *v51.2/F5L-F11L is significantly different

280   to all other viruses (p<0.05 for v51.2/F5L, all others p<0.001) One-way ANOVA (n=50) and Tukey

281   pairwise test). (c) Fluorescent foci of MVA/F5LGb and v51.2/F5LGb formed on BS-C-1 under

282   semisolid media at 72h.p.i. (100× final magnification). (d-e) Replication analysis in BS-C-1. Data

283   are mean ± SEM of three independent wells (d) Multiple step growth analysis (m.o.i.=0.01). (e)

284   Single step growth analysis (m.o.i.=5). (f) Multiple step growth analysis (m.o.i.=0.01, BS-C-1) of

285   MVA and two independent rescues of *F5L* in MVA (MVA/F5LGb #1 and MVA/F5LGb #2). Data

286   expressed as fold increase (mean ± SEM of three independent wells). (g) Disposition of genes

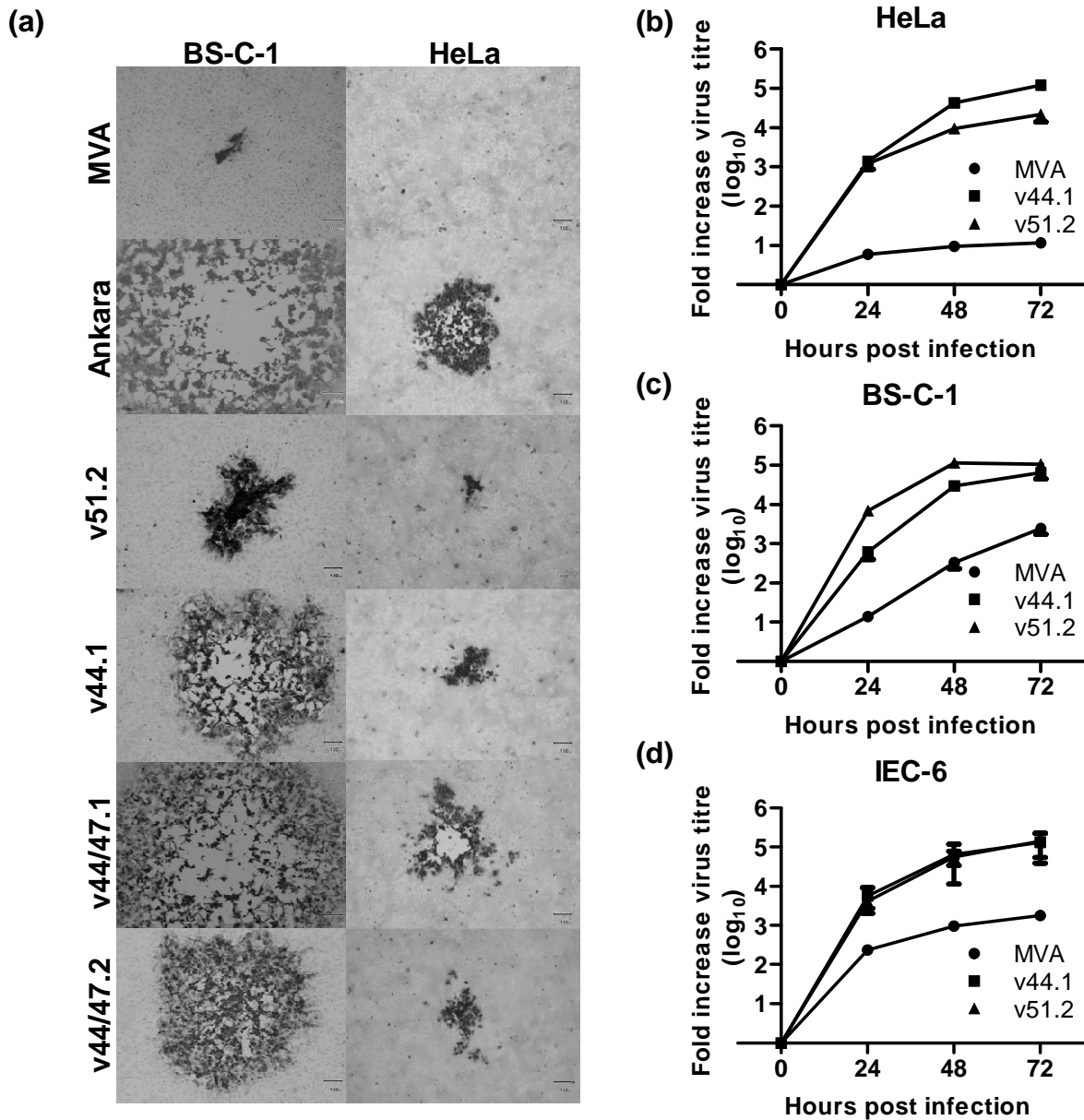287   associated with plaque phenotype in rescued MVAs.

Fig 1: Rescued MVAs show a range of plaque size and morphology on BS-C-1 and HeLa cells. (a) Representative plaques formed by the viruses shown on BS-C-1 or HeLa cells under semisolid media. Cells were immunostained at 72 h.p.i. (100× final magnification, scale bars 100μm). (b-d) Multiple step growth analysis (m.o.i.=0.01) in (b) HeLa, (c) BS-C-1, or (d) IEC-6. Cells were incubated for 1h with virus then washed and fresh media added. 0 h.p.i. samples were harvested immediately after addition of fresh media. Cell associated virus collected at 24, 48 and 72h.p.i. was titrated and divided by virus titre present after absorption. Data points represent mean ± SEM of three independent wells.
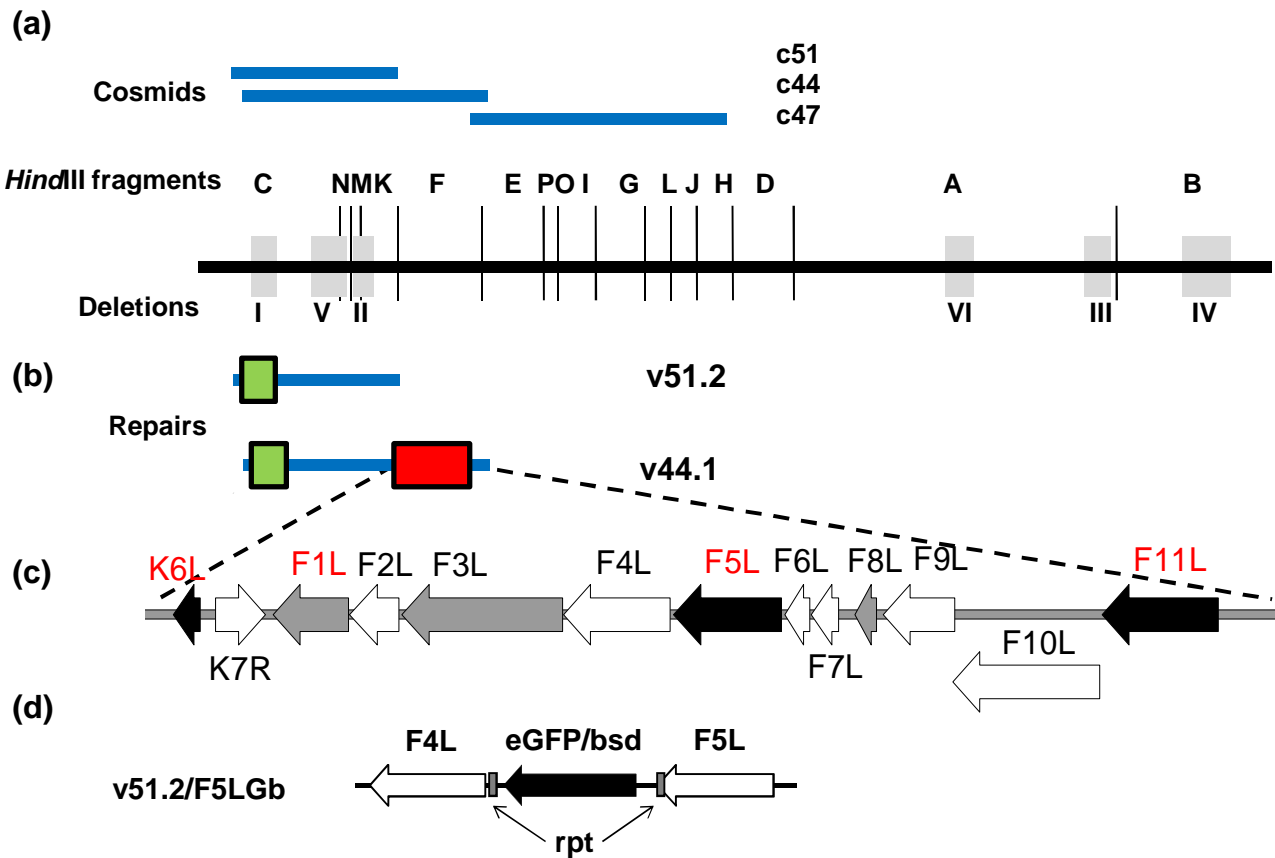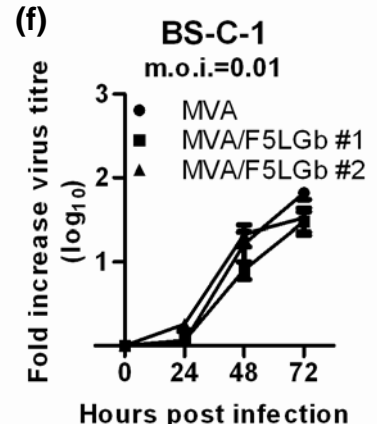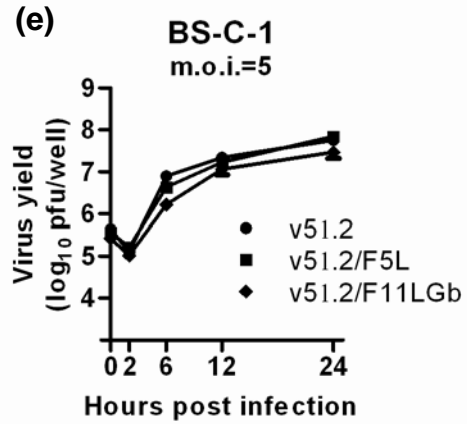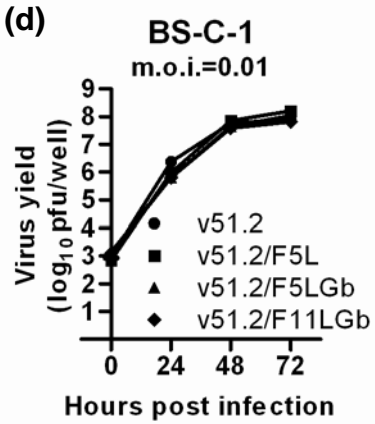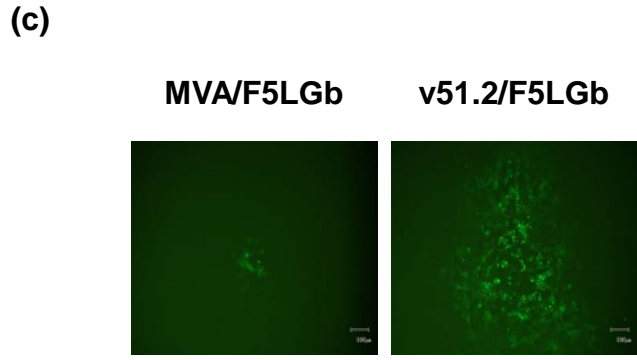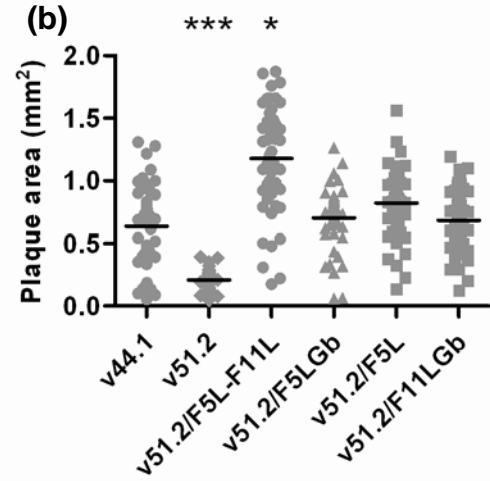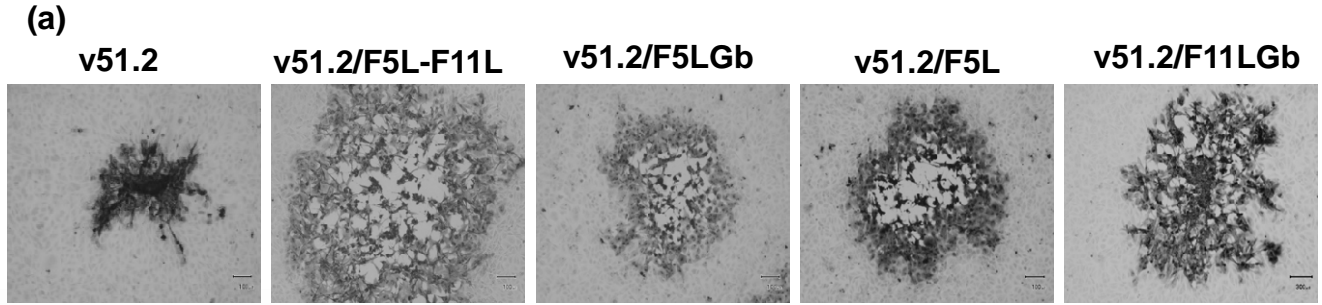
Fig 2: MVA genome and repairs in v51.2 and v44.1 (a) Map of the MVA genome showing the location of the six major deletions, indicated by grey boxes and the approximate location of cosmids used to make the rescued MVAs (blue bars). *Hind*III fragments of Copenhagen are marked for reference. (b) Approximate locations of identified repairs in v51.2 and v44.1 are indicated in relation to the rescuing cosmid (blue bar). Repairs surrounding deletion I in v51.2 and v44.1 are indicated by the green boxes and a second repair, unique to v44.1, by the red box. (c) Mapping of the repair unique to v44.1: ORFs shown in black are truncated in MVA compared with CVA. Genes shown in white are identical between CVA and MVA. Genes shown in grey contain small mutations in MVA (4 aa deletions in F1L and F3L, single aa substitution in F8L). Genes labelled in red were sequenced in v44.1 and v51.2 to identify the extent of the repair. For these four genes v44.1 matches Ankara not v51.2 or MVA.(d) Structure of *F5L* region in v51.2/F5LGb. *F5L* is followed by *GFP/bsd* driven by a strong synthetic promoter (not shown) and flanked by repeated sequences (rpt) to preserve the promoter for *F4L*. The structure of *F11L* in v51.2/F11LGb was similar.

(a)

v51.2 v51.2/F5L-F11L v51.2/F5LGb v51.2/F5L v51.2/F11LGb

(b)

*** *

Plaque area (mm^2)

v44.1 v51.2 v51.2/F5L-F11L v51.2/F5LGb v51.2/F5L v51.2/F11LGb

(c)

MVA/F5LGb v51.2/F5LGb

(d)

BS-C-1
m.o.i.=0.01

Virus yield (log$_{10}$ pfu/well)

- v51.2
- v51.2/F5L
- v51.2/F5LGb
- v51.2/F11LGb

Hours post infection

(e)

BS-C-1
m.o.i.=5

Virus yield (log$_{10}$ pfu/well)

- v51.2
- v51.2/F5L
- v51.2/F11LGb

Hours post infection

(f)

BS-C-1
m.o.i.=0.01

Fold increase virus titre (log$_{10}$)

- MVA
- MVA/F5LGb #1
- MVA/F5LGb #2

Hours post infection

(g)

C NM K F E P O

left end of genome

	C2L	F5L	F11L	O1L	
v44.1	✕	✓	✓	✕	Medium plaque
v44.2	✓	✓	✓	✕	Similar morphology to v44.1, larger
v51.1	✓	✕	✕	✕	Small pile of cells
v51.2	✕	✕	✕	✕	Small pile of cells, similar to v51.1
v51.2/F5L-F11L	✕	✓	✓	✕	Similar morphology to v44.1, larger
v51.2/F5L	✕	✓	✕	✕	Medium plaque, similar to v44.1
v51.2/F11LGb	✕	✕	✓	✕	Larger pile of cells (no clearance)
v44/47.1	✓	✓	✓	✓	Large plaque, but smaller than Ankara
v44/47.2	✓	✕	✓	✓	Medium plaque, decreased clearance

Fig 3: Restoration of *F5L* or *F11L* to v51.2 alters plaque morphology but not replication.
(a-b) Plaques formed on BS-C-1 cells under semisolid media by the recombinant viruses shown were immunostained 72 h.p.i. (a) Representative plaques, original magnification 100× (scale bar 100μm). (b) Areas of individual plaques are plotted with the average shown by the solid line. (*** v51.2 significantly different to all other viruses (p<0.001), *v51.2/F5L-F11L is significantly different to all other viruses (p<0.05 for v51.2/F5L, all others p<0.001) One-way ANOVA (n=50) and Tukey pairwise test). (c) Fluorescent foci of MVA/F5LGb and v51.2/F5LGb formed on BS-C-1 under semisolid media at 72h.p.i. (100× final magnification). (d-e) Replication analysis in BS-C-1. Data are mean ± SEM of three independent wells (d) Multiple step growth analysis (m.o.i.=0.01). (e) Single step growth analysis (m.o.i.=5). (f) Multiple step growth analysis (m.o.i.=0.01, BS-C-1) of MVA and two independent rescues of *F5L* in MVA (MVA/F5LGb #1 and MVA/F5LGb #2). Data expressed as fold increase (mean ± SEM of three independent wells). (g) Disposition of genes associated with plaque phenotype in rescued MVAs.