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Pathogenic variants in *SLF2* and *SMC5* cause segmented chromosomes and mosaic variegated hyperploidy.

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98

99   **Abstract**

100   Embryonic development is dictated by tight regulation of DNA replication, cell division and

101   differentiation. Mutations in DNA repair and replication genes disrupt this equilibrium, giving rise to

102   neurodevelopmental disease characterised by microcephaly, short stature and chromosomal breakage.

103   Here, we identify biallelic variants in two components of the RAD18-SLF1/2-SMC5/6 genome stability

104   pathway, *SLF2* and *SMC5*, in 11 patients with microcephaly, short stature, cardiac abnormalities and

105   anaemia. Patient-derived cells exhibit a unique chromosomal instability phenotype consisting of

106   segmented and dicentric chromosomes with mosaic variegated hyperploidy. To signify the importance

107   of these segmented chromosomes, we have named this disorder Atelís (meaning - incomplete)

108   Syndrome. Analysis of Atelís Syndrome cells revealed elevated levels of replication stress, partly due

109   to a reduced ability to replicate through G-quadruplex DNA structures, and also loss of sister chromatid

110   cohesion. Together, these data strengthen the functional link between SLF2 and the SMC5/6 complex,

111   highlighting a distinct role for this pathway in maintaining genome stability.

112

Introduction

Despite the fundamental nature of DNA replication and cell division, inherited variants in genes involved in these processes are an underlying cause of human disease. Whilst these syndromes usually display unique clinical features that define them diagnostically, they typically exhibit common neurodevelopmental deficits, such as severe microcephaly and pre- and post-natal growth retardation[1-3]. As such, many of these syndromes can be collectively referred to as microcephalic dwarfism (MD) disorders. This constellation of conditions includes Meier-Gorlin Syndrome, Seckel Syndrome Spectrum Disorders, Bloom Syndrome and Microcephalic Osteodysplastic Primordial Dwarfism type II and can be broadly classified as having deficiencies in one of three cellular processes: DNA replication, DNA repair, and mitotic cell division[1-4]. Although mechanistically distinct, the common clinical phenotypes exhibited by these diseases are thought to result from a reduction in cellular proliferation and/or excessive cell death in the developing embryo, which reduces the number of cells available to maintain normal foetal growth[5]. Cells from these patients often exhibit signs of increased genome instability, such as micronuclei and/or elevated chromosome breakage. A distinct subgroup of these syndromes exhibit rare cytogenetic anomalies, for example, mosaic variegated aneuploidy syndrome (MVA)[6-8] caused by variants in the spindle assembly checkpoint genes *BUB1B*, *CEP57* and *TRIP13*, or railroad chromosomes and premature chromatid separation (PCS) associated with Warsaw Breakage Syndrome (WABS) and Cornelia de Lange syndrome, caused by variants in the helicase DDX11 and components of SMC1/3 cohesin complex respectively[9,10]. Whilst, the presence of these chromosomal abnormalities is a useful diagnostic tool they can also help dissect the cellular mechanisms underlying the disease pathology.

Here, we report 11 patients with a neurodevelopmental disorder overlapping clinically with MVA and Fanconi Anaemia (FA) with pathogenic variants in SLF2 and SMC5, two components of the recently discovered RAD18-SLF1/2-SMC5/6 genome stability pathway[11]. The precise function of the SMC5/6 complex remains enigmatic, however, it has been linked to a number of fundamental processes, including DNA transcription, DNA replication, DNA repair and chromosome segregation[12,13]. Evidence suggests that the primary function of this complex occurs during DNA replication to stabilise stalled forks, suppress the activity of pro-recombination factors and promote efficient replication through difficult-to-replicate and/or repetitive regions of the genome, such as rDNA and telomeres[14]. In contrast,

142 the function of SLF1 and SLF2 remain unclear, other than a reported role in recruiting the SMC5/6

143 complex to sites of DNA damage[11].

144   Analysis of SLF2 and SMC5 patient-derived cell lines revealed spontaneous replication stress

145 and multiple mitotic abnormalities that give rise to a unique, diagnostically relevant, genome instability

146 phenotype consisting of segmented, dicentric and railroad chromosomes, and mosaic variegated

147 hyperploidy (MVH). The underlying basis for this chromosomal instability is not fully understood, but our

148 data suggest that it may arise, in part, from the failed resolution of aberrant DNA structures during S-

149 phase, such as G-quadruplexes (G4), potentially leading to a combination of under-replicated DNA and

150 unresolved recombination intermediates persisting through to mitosis. Together, these data

151 demonstrate that despite a hitherto unknown role as a core component of the SMC5/6 complex, SLF2

152 is essential for the SMC5/6 cohesin-like complex to maintain genome stability by regulating both DNA

153 replication and cell division.

154

155 **Results**

156 **Patients with microcephaly and short stature have biallelic *SLF2* (*FAM178A*) and *SMC5* variants**

157   Whole exome sequencing (WES) was carried out on six patients (P1, P2, P3, P4-1, P4-2, P5

158 and P6) from five families, presenting with microcephaly, short stature, mild to severe developmental

159 delay and spontaneous chromosome breakage. After aligning WES reads to the reference genome,

160 variant calling, and filtering for rare variants (MAF <0.005), analysis under a recessive model of

161 inheritance identified biallelic variants in *SLF2* (*FAM178A*) in all six patients. All identified *SLF2* variants

162 segregated amongst family members (with the exception of patients P1 and P5 where parental material

163 was unavailable) and were present at a frequency of <0.5% in the gnomAD database (Figure 1a, 1c;

164 Supplementary Table 1-7; Supplementary Figure 1a). Comparative genomic hybridisation (CGH) array

165 analysis carried out on gDNA from patient P5 confirmed the homozygosity of the identified *SLF2* variant.

166   Given that SLF2 had been identified previously as part of the RAD18-SLF1/2-SMC5/6 genome

167 stability pathway[11], we hypothesised that variants in other components of this pathway may also give

168 rise to a similar neurodevelopmental disorder. By querying gene matching platforms, four patients

169 exhibiting microcephaly and growth retardation that had undergone WES were identified to carry

170 biallelic variants in *SMC5*: patient P7 (c.1110_1112del; p.Arg372del, c.1273C>T; p.Arg425Ter) and

171 patients P8, P9-1 and P9-2 (c.2970C>G; p.His990Asp) (Figure 1a, 1c; Supplementary Table 1;

172   Supplementary Table 8-10; Supplementary Figure 1b). All variants were verified by Sanger sequencing,

173   segregated amongst family members in an autosomal recessive paradigm and were present at a

174   frequency of <0.5% in gnomAD.

175

176   **SLF2 and SMC5 variants give rise to neurodevelopmental abnormalities, cardiac defects and**

177   **anaemia.**

178   All individuals with *SLF2* and *SMC5* variants presented with a similar clinical phenotype,

179   including marked microcephaly (-3.57 to -11.88 SD) and a reduction in height (-2.19 to -8.24 SD) (Figure

180   1b; Supplementary Table 1). Moreover, the majority of patients also exhibited a developmental delay

181   along with learning difficulties. Mild skeletal defects (i.e. clinodactyly), skin hyperpigmentation and

182   ocular abnormalities were present in several patients (Supplementary Table 1). Notably, two of seven

183   SLF2 patients (P4-1, P5) and all four SMC5 patients (P7, P8, P9-1 and P9-2) displayed cardiac defects

184   (Supplementary Table 1), such as atrial or ventricular defects, a phenotype commonly observed in

185   patients with cohesinopathies[15,16] but not DNA replication disorders. Furthermore, five of eleven patients

186   (P3, P4-1, P4-2, P5, P9-2) also developed anaemia, with one of these patients (P9-2) subsequently

187   developing myelodysplastic syndrome (Supplementary Table 1). This, coupled with other clinical

188   features, could potentially result in future cases being mistakenly diagnosed with an atypical form of FA

189   in the absence of a clear genetic diagnosis using WES. This is particularly relevant since components

190   of the SMC5/6 complex have been previously shown to functionally interact with the FA pathway to

191   repair DNA damage[17]. Only one patient (P3) developed severe pulmonary disease similar to patients

192   with variants in the SMC5/6 complex subunit NSMCE3[18,19], whereas insulin-resistant diabetes and

193   metabolic dysfunction, which are characteristic to patients with *NSMCE2* variants were absent among

194   this cohort[20]. Collectively, these clinical and genetic observations support the premise that variants in

195   *SLF2* and *SMC5* cause microcephaly and short stature associated with cardiac defects and the

196   development of anaemia.

197

198   **SLF2 and SMC5 variants compromise protein stability, interactions with other components of**

199   **the RAD18-SLF1/2-SMC5/6 pathway and recruitment to sites of DNA damage**

200   To determine the pathogenicity of the identified patient variants, we carried out western blotting

201   on extracts from SLF2 patient-derived cell lines (SLF2-P1, SLF2-P2, SLF2-P3 and SLF2-P4-1) to

202   ascertain if SLF2 protein abundance or stability was compromised. Notably, all four of the SLF2 mutant

203   patient cell lines examined exhibited a reduction or absence of detectable full length SLF2 protein whilst

204   maintaining wild type (WT) levels of RAD18, SMC5, and SMC6 protein (Figure 2a). SLF1 protein level

205   was not tested due to the absence of an available antibody.

206   We next investigated the *SLF2* variants in patients P2 and P3 in more detail. Analysis of cDNA

207   from the SLF2-P3 cell line demonstrated that the synonymous homozygous variant c.3330G>A

208   (p.Arg1110Arg), disrupted splicing leading to an in-frame deletion of exon 17 (Supplementary Figure

209   2a-b). We then analysed the impact of the c.3486G>C (p.Gln1162His) variant, present in patient P2,

210   on splicing. Multiple *SLF2* transcripts are annotated in the human genome and although c.3486G>C

211   (p.Gln1162His) introduces a nonsynonymous change in the two longest transcripts (NM_018121 and

212   NM_001136123), it only affects mRNA splicing of the most abundant *SLF2* transcript (NM_018121) by

213   impairing the exon 19 splice donor splice site (Supplementary Figures 2c, 3a-e). The p.(Gln1162His)

214   variant also displayed compromised protein stability when expressed transiently indicating that this

215   variant disrupts both mRNA and protein stability (Supplementary Figure 3f). Together, these data

216   suggest that most of the identified *SLF2* variants have an adverse effect on protein stability.

217   In contrast, analysis of SMC5 patient cell lines revealed that the homozygous p.(His990Asp)

218   variant present in patients P8, P9-1 and P9-2 had little detectable impact on the protein stability of

219   SMC5, or RAD18, SLF2, and SMC6 (Figure 2b). Only a cell line derived from patient P7 exhibited a

220   reduced abundance of SMC5 protein, presumably due to the presence of a nonsense variant

221   (p.Arg425Ter) on one of the *SMC5* alleles. As loss of Smc5 is embryonically lethal[21], it is possible that

222   the *SMC5* variants are hypomorphic and that significant disruption of SMC5 protein stability to the extent

223   observed with the SLF2 variants is incompatible with life.

224   SLF1 and SLF2 have been identified as bridging factors between RAD18 and the SMC5/6

225   complex at sites of stalled replication[11]. To address whether the SLF2 and SMC5 variants compromised

226   their ability to bind components of the RAD18-SLF1/2-SMC5/6 pathway, we initially mapped the binding

227   sites of RAD18, SLF1 and SMC6 on SLF2. Using co-immunoprecipitation analysis with tagged proteins,

228   we determined that the binding of RAD18 and SLF1 to SLF2 requires the C-terminal 471 amino acids

229   (aa702-1173), which also overlapped with the SMC6 binding site located at amino acids 589-810

230   (Supplementary Figure 4a-d). All patient-associated variants in SLF2, with the exception of

231   p.(Gln1162His), are located within or truncate the SLF1/RAD18 binding domain of SLF2 (Figure 1c).

232 Consistent with SLF1 binding being essential for SLF2 to mediate bridging between RAD18 and the

233 SMC5/6 complex, co-immunoprecipitation studies using extracts from hydroxyurea (HU) treated SLF2

234 patient-derived LCLs revealed a failure of all cell lines tested to co-purify SMC6 with RAD18 (Figure

235 2c). Furthermore, all SLF2 mutant proteins, with the exception of p.(Gln1162His), failed to or exhibited

236 a reduced ability to, be recruited to sites of DNA damage induced by laser micro-irradiation

237 (Supplementary Figure 3e).

238   We next extended the co-immunoprecipitation analysis to include SMC5 patient LCLs (Figure

239 2d). The interaction between RAD18 and SMC6 in SMC5-P8 and SMC5-P9-1 cells was observed to be

240 at WT levels, suggesting that p.(His990Asp) had no discernible impact on the integrity of the RAD18-

241 SLF1/2-SMC5/6 complex, whereas the association of RAD18 with SMC6 was partially affected in

242 SMC5-P7 cells. However, both the p.(Arg372del) and p.(His990Asp) SMC5 mutants failed to re-localise

243 efficiently to sites of laser micro-irradiation induced damage, with the latter being more severely affected

244 (Supplementary Figure 4f). These observations indicate that whilst these variants largely do not appear

245 to compromise their binding to components of the RAD18-SLF1-SLF2-SMC5/6 pathway, they do affect

246 their re-localisation to and/or retention at sites of damage.

247   To gain insight into why the SMC5 mutants affected stability of the SMC5/6 complex at sites of

248 damage, we carried out co-immunoprecipitation analysis to assess if these mutations affected binding

249 to other components of the complex. Interestingly, whilst the p.(His990Asp) mutation did not

250 significantly affect binding to other components of the SMC5/6 complex, the p.(Arg372del) significantly

251 compromised binding to SLF2, SMC6 and NSMCE2 (Figure 2e). Moreover, endogenous NSMCE2

252 exhibited reduced binding to SMC5 in cells from patient SMC5-P7 (Figure 2f). Consistent with these

253 observations, the Nse2 binding site on yeast Smc5 lies in close proximity to Lys368, which is the yeast

254 functional equivalent of human SMC5 Arg372 (Supplementary Figure 5). This suggests that the failure

255 of the p.(Arg372del) mutant SMC5 to be recruited to sites of laser damage may be due to this mutation

256 compromising the binding of other key components of the SMC5/6 complex.

257   To explore the possibility that the p.(His990Asp) may have a deleterious impact on the structure

258 of the SMC5/6 complex, we compared the AlphaFold model for human SMC5 to the X-ray crystal

259 structures for the head domain of *Pyrococcus furiosus* Rad50 (*Pf.*Rad50) in both the unliganded and

260 ATP-bound forms[23]. Notably, His990 lies just upstream of the ATP-binding cassette (ABC) signature

261 motif of Smc5 (Supplementary Figure 6a), a region of the protein implicit in both binding ATP and

262   mediating the complex set of conformational changes that occur when SMC proteins bind nucleotide[22].

263   Interestingly, His990 sits in a position functionally equivalent to Phe791 of *Pf*.Rad50 – a residue known

264   to interact directly with the adenine moiety of bound ATP[23]. Whilst mutation of His990 to aspartic acid

265   would appear to be tolerated and unlikely to cause any gross-misfolding of the protein, as judged by

266   the lack of steric clashes produced by the mutation (Supplementary Figure 6b), it removes an aromatic

267   amino acid and replaces it with one carrying a negative charge. As such, this would alter the overall

268   charge of a region that normally functions to accept the adenine moiety. Therefore, it is likely that the

269   p.(His990Asp) mutation perturbs the ability of the complex to either bind or turnover ATP, in turn

270   affecting its association with, or retention on chromatin[24].

271

272   **Cell cycle arrest and increased apoptosis in the developing brain underlies the development of**

273   **microcephaly in zebrafish lacking *slf2* and *smc5***

274   To gain insight into how SLF2 and SMC5 patient associated variants affect neurodevelopment,

275   we utilised CRISPR-Cas9 genome-editing to ablate the single zebrafish orthologs of each of *slf2* and

276   *smc5* in zebrafish embryos. Single guide (sg) RNAs targeting the primary isoforms of *slf2* and *smc5*

277   (Supplementary Figure 7a, 7f) were injected, with or without recombinant Cas9 protein, into -

278   *1.4col1a1:egfp* reporter embryos at the single-cell stage, which were allowed to develop until 3 days

279   post-fertilisation (dpf) (Supplementary Figure 7b-c, 7g-h). This reporter allows visualisation of

280   craniofacial patterning during embryonal development[25]. Bright field lateral images were acquired to

281   measure head size and ventral fluorescent images of GFP-positive cells allowed visualisation of the

282   pharyngeal skeleton. Similar to the clinical phenotype exhibited by SLF2 and SMC5 patients, zebrafish

283   embryos lacking *slf2* and *smc5* displayed a significant reduction in head size and aberrant craniofacial

284   patterning, as indicated by a broadening of the angle of the ceratohyal cartilage; a major mandibular

285   structure (Figure 3a-f). Furthermore, unlike *smc5*, which is an essential gene[21], we were able to

286   generate stable F2 *slf2* mutants possessing a frameshifting 8 bp deletion allele in *slf2* exon 7

287   (c.515_522del; p.Ser172_Ser174fsTer191; Supplementary Figure 7d-e). Consistent with our

288   observations from F0 embryos injected with sgRNA and Cas9, stable F2 *slf2* null mutants also exhibited

289   microcephaly and aberrant craniofacial patterning (Figure 3g).

290   To validate these findings, we used morpholinos (MO) to suppress the expression of *slf2* and

291   *smc5* in zebrafish embryos. Splice blocking MO targeting the single zebrafish ortholog of each gene,

292   *slf2* (exon 11) and *smc5* (exon3), were designed and depletion of *slf2* and *smc5* mRNA was confirmed

293   by RT-PCR after injection into zebrafish larvae (Supplementary Figure 8a-b). MO were injected into -

294   *1.4col1a1:egfp* reporter embryos at the single-cell stage. Injected embryos were reared to 3 dpf and

295   then bright field images were acquired to measure head size and ventral fluorescent images of GFP-

296   positive cells to visualise the pharyngeal skeleton. Comparable to our observations from the zebrafish

297   embryos lacking *slf2* and *smc5*, zebrafish embryos depleted of *slf2* and *smc5* using MO also displayed

298   a significant reduction in head size and aberrant craniofacial patterning in the pharyngeal skeleton

299   (Supplementary Figure 8e-h, Supplementary Figure 9a-f), which could both be rescued by re-

300   expression of WT human *SLF2* or *SMC5* mRNA.

301   To confirm the pathogenicity of the *SMC5* disease associated variants we utilised our *smc5*

302   morphant zebrafish model to ascertain whether the three patient-associated *SMC5* variants could

303   rescue the developmental abnormalities caused by loss of *smc5* expression. Neither the p.(Arg425Ter),

304   p.(Arg372del) nor p.(His990Asp) variants could complement the reduced head size and increased

305   ceratohyal angle resulting from *smc5* depletion (Supplementary Figure 9g-i), reinforcing that they confer

306   a loss of function effect. In contrast, both the head size and ceratohyal angle could be restored to normal

307   following expression of WT human *SMC5* or a polymorphic *SMC5* variant, p.(Arg733Gln), identified

308   from gnomAD.

309   To investigate the two principal underlying causes of microcephaly, slowed cell cycle

310   progression and/or increased apoptosis in the developing brain[2,26-28], fixed wholemount *slf2* and *smc5*

311   depleted zebrafish embryos were stained with markers of cell cycle stage (G2/M: phospho-histone H3

312   serine-10) and apoptosis (TUNEL). F0 CRISPR embryos injected with either *slf2* or *smc5* sgRNA with

313   recombinant Cas9 (Figure 4) exhibited a pronounced increase in both phospho-histone H3 and TUNEL

314   staining in the developing brain when compared to control zebrafish. Importantly, this phenotype was

315   recapitulated in zebrafish embryos transfected with *slf2* or *smc5* MO, which could be complemented by

316   re-expression of the orthologous WT human mRNA (Supplementary Figure 10). Together, these in vivo

317   data confirm that a functional RAD18-SLF1/2-SMC5/6 pathway is required for normal development of

318   the brain and cartilaginous structures, and compromising this pathway triggers a G2/M cell cycle arrest

319   and the onset of apoptosis leading to microcephaly.

320

321   **SLF2/SMC5 mutant patient-derived cell lines exhibit increased spontaneous replication stress**

322   Although the SMC5/6 complex has been implicated in regulating numerous DNA repair and

323   replication pathways, it is thought that its primary function is to promote efficient replication[14,29].

324   Therefore, we used DNA fibre analysis to study the impact of SLF2 and SMC5 variants on replication

325   dynamics. All *SLF2* and *SMC5* mutant LCLs examined exhibited a significant increase in spontaneous

326   replication fork stalling and fork asymmetry comparable to that observed in an LCL derived from an

327   ATR-Seckel Syndrome patient (Figure 5a-d). Importantly, this increased spontaneous replication fork

328   stalling was also observed in patient-derived fibroblasts and could be suppressed by re-expressing WT

329   *SLF2* or *SMC5* (Figure 5e-f; Supplementary Figure 11a-b). Unlike the ATR-Seckel cell line, all the SLF2

330   mutant LCLs and one of the SMC5 mutant LCLs exhibited WT levels of replication fork speed

331   (Supplementary Figure 11c-d). In contrast, LCLs carrying the homozygous p.(His990Asp) exhibited a

332   moderate reduction in replication fork speed.

333   To confirm these observations, we used CRISPR-Cas9 gene editing in U-2 OS cells to generate

334   SLF2 knockout clones. Despite several attempts we were unable to generate complete SLF2 knockout

335   clones. Rather, we generated two hypomorphic (HM) clones, each with one expressed mutant allele of

336   *SLF2* in conjunction with one or more truncating mutant alleles: SLF2 HM cl.1 (p.Asn411Lysins16,

337   p.Ser403Ter, p.Asn411LysfsTer3) and SLF2 HM cl.2 (p.Asp398_Ser404del, p.Ser403ThrfsTer14).

338   These clones were subsequently complemented by re-expressing WT *SLF2* (Supplementary Figure

339   12). Importantly, DNA fibre analysis of these SLF2 HM clones demonstrated that the vector

340   complemented SLF2 HM cell lines exhibited significantly elevated levels of spontaneous fork stalling

341   compared to the WT SLF2 complemented clones (Figure 5g).

342   Since spontaneous replication stress exhibited by cells can be attributed to defective ATR-

343   dependent DNA damage signalling, we used DNA fibre analysis and western blotting to monitor

344   activation of the ATR-dependent stress response[30,31]. In contrast to the ATR-Seckel syndrome cell line,

345   all the SLF2 or SMC5 patient cell lines were capable of activating ATR or the intra-S phase checkpoint

346   in response to HU and MMC (Supplementary Figure 11e-f, Supplementary Figure 13) indicating that

347   dysregulation of the ATR stress response pathway does not account for the observed DNA replication

348   defects. This is consistent with previous work demonstrating that loss of the SMC5/6 pathway does not

349   affect activation of the ATR-dependent DDR[17].

350   We next investigated the cellular impact of the increased spontaneous replication fork instability

351   observed in the patient cell lines using different markers of replication stress. Significantly, both SLF2

352   and SMC5 patient cell lines exhibited elevated signs of spontaneous replication stress including the

353   presence of DNA double strand breaks (DSBs) in S-phase cells (53BP1 foci in EdU positive cells), an

354   increased frequency of mitotic cells undergoing mitotic DNA synthesis (MiDAS), elevated levels of

355   53BP1 G1 bodies and the formation of micronuclei (Figure 6a-d, Supplementary Figure 14a-d)[17,29].

356   Crucially, all these phenotypes could be complemented by re-expressing either WT *SLF2* or *SMC5*

357   (Figure 6). Moreover, the U-2 OS SLF2 HM cell lines also exhibited elevated levels of micronuclei

358   compared to the corrected WT SLF2 expressing clones (Figure 6e).

359

360   **Hypomorphic variants in *SLF2* and *SMC5* are associated with mitotic abnormalities, segmented**

361   **chromosomes, cohesion defects and mosaic variegated hyperploidy**

362   Consistent with the elevated levels of spontaneous replication stress, LCLs derived from SLF2

363   and SMC5 mutant patients all exhibited increased levels of chromosomal aberrations (such as

364   chromosome and chromatid gaps/breaks and chromosome radials) comparable to that observed in an

365   ATR Seckel Syndrome LCL (Figure 6f-g). Notably, this phenotype was not significantly exacerbated by

366   exposure to either APH or MMC, unlike LCLs from an ATR-Seckel Syndrome patient (Supplementary

367   Figure 15a-b). Importantly, the elevated spontaneous levels of chromosomal aberrations in the

368   SLF2/SMC5 patient fibroblasts and the U-2 OS SLF2 HM cells, was rescued by re-expression of either

369   WT *SLF2* or *SMC5* (Figure 6h-i).

370   In addition to the spontaneous chromosomal aberrations, metaphase spread analysis of both

371   the peripheral blood and patient-derived LCLs of SLF2 and SMC5 patients revealed that a significant

372   subset of cells exhibited large increases in chromosome numbers, with some metaphases having >100

373   chromosomes (Figure 7a; Supplementary Figure 16a-b; Supplementary Figure 17a). Unlike MVA,

374   which typically involves the loss/gain of small numbers of chromosomes, the cytogenetic abnormality

375   observed in SLF2 and SMC5 patient cells predominantly involved huge chromosomal gains. Therefore,

376   we have termed this cytogenetic abnormality mosaic variegated hyperploidy (MVH), i.e. chromosome

377   number >46.

378   To investigate the cause of the MVH, we explored whether SLF2 or SMC5 patient-derived cell

379   lines exhibited spontaneous mitotic abnormalities. Both SLF2 and SMC5 patient fibroblast cell lines,

380   and U-2 OS SLF2 HM cells, displayed a significant increase in mitotic cells with lagging chromosomes

381   in empty vector complemented cells compared to cells re-expressing WT protein (Figure 7b-d),

382   consistent with previous reports[17,29,32]. Additionally, when we examined the origins of these lagging

383   chromosomes/micronuclei using CENPA as a marker of centromeres, it was evident that a significant

384   proportion of the micronuclei were positive for CENPA, suggesting that they could have resulted from

385   failed mitotic segregation (Supplementary Figure 16c-d). This is supportive of the RAD18-SLF1/2-

386   SMC5/6 pathway playing an important role in promoting proper chromosomal segregation.

387   Since SMC5/6 forms a cohesin-like complex and has been implicated in facilitating centromeric

388   and sister chromatid cohesion[21,32-35], we analysed metaphase spreads from SLF2 and SMC5 patient-

389   derived cells for the presence of cohesion defects. SLF2 and SMC5 peripheral blood lymphocytes

390   showed loss of sister chromatid cohesion as evidenced by the presence of railroad chromosomes

391   (Figure 7e; Supplementary Figure 17b). Moreover, SLF2 and SMC5 patient-derived LCLs exhibited

392   PCS after treatment with the proteasome inhibitor MG132, which is known to induce cohesion fatigue

393   by preventing the metaphase-to-anaphase transition[36] (Figure 7f). Together, these observations

394   suggest that the MVH characteristic to SLF2 and SMC5 patient cells may also be caused by PCS

395   resulting from cohesion fatigue.

396   However, given the extent of the karyotypic abnormalities it seemed plausible that other cellular

397   defects may contribute to the large increases in chromosome number seen in SLF2 and SMC5 mutant

398   cell lines in addition to PCS. Replication stress can trigger centrosome amplification via fragmentation

399   of the pericentriolar material (PCM)[37] or premature centriole disengagement, which can lead to mitotic

400   arrest and aneuploidy-induced cell death and microcephaly[38]. To investigate whether centrosome

401   abnormalities could contribute to the cellular pathology associated with SLF2 and SMC5 dysfunction,

402   patient-derived cell lines were subjected to immunofluorescence with antibodies to PCNT1 (a

403   component of the PCM) and mitosin/CENPF (marker of S/G2 cells) before and after incubation with

404   aphidicolin (APH). Notably, following APH exposure a significant proportion of S/G2 cells possessed

405   more than two centrosomes (Figure 7g). We also observed that APH treatment had a profound effect

406   on mitosis with >10-50% of SLF2 and SMC5 patient-derived LCLs exhibiting multi-polar spindles during

407   mitosis (Figure 7h, Supplementary Figure 16e). This increase in centrosome number and multi-polar

408   spindles is not due to higher levels of replication stress in the APH treated patient cells as quantification

409   of APH-induced G1 53BP1 bodies revealed no difference between empty vector and WT SLF2/SMC5

410   complemented cells (Figure 7i). Therefore, it is likely that the MVH observed in SLF2 and SMC5 patient

411   cells arises as a consequence of multiple defects including unresolved replication stress, PCS,

412   chromosome mis-segregation and centrosome amplification.

413

414   **SLF2/SMC5 mutant cells are unable to replicate efficiently in the presence of stabilised G-**

415   **quadruplex structures.**

416   During our analysis of metaphase spreads of peripheral blood lymphocytes from SLF2 and

417   SMC5 patients, we noted that among the increased levels of spontaneous chromosomal damage, two

418   distinct types of chromosome abnormality were evident (Figure 8a; Supplementary Figure 18). The first

419   type of abnormal chromosome, which we termed segmented chromosomes, contained one or more

420   chromosome gaps/breaks along the body of the chromosome (type 1). Type 1 segmented

421   chromosomes with two or more gaps/breaks were particularly evident in SLF2-P1 and SLF2-P3, whilst

422   most of the segmented chromosomes in SLF2-P2 and SMC5-P7 possessed one gap/break. The

423   second type of abnormal chromosomal structure resembled a dicentric chromosome, which was

424   confirmed by the presence of two centromeres using centromere specific FISH probes (type 2) (Figure

425   8b).

426   The type 1 segmented chromosomes were reminiscent of the chromosomal abnormalities

427   resulting from combined inactivation of GEN1 and either MUS81 or SLX4, suggesting that they may be

428   caused by an inability to resolve recombination intermediates[39,40]. Accordingly, both SLF2 and SMC5

429   patient-derived cell lines exhibited elevated levels of recombination as indicated by increased levels of

430   spontaneous RAD51 foci and sister chromatid exchanges (SCEs) in the patient-derived fibroblasts and

431   LCLs respectively (Figure 8c, Supplementary Figure 19a-b, Supplementary Figure 15c-d). This is in line

432   with previous work demonstrating a role for the SMC5/6 complex in resolving recombination

433   intermediates[41-44]. We also observed an increased frequency of telomeric SCEs in SLF2 mutant LCLs

434   (Supplementary Figure 19c), which could, in part, contribute to the generation of the observed dicentric

435   chromosomes. To investigate whether the spontaneous chromosomal aberrations observed in

436   SLF2/SMC5 mutant cells could arise as a consequence of the presence of unresolved HR

437   intermediates, we examined the effect of stably expressing the bacterial Holliday junction resolvase,

438   RusA, in patient-derived cell lines on genome stability[40]. In line with SLF2 and SMC5 dysfunction

439   causing unresolved HR intermediates to accumulate and this leading to increased genome instability,

440   expression of WT RusA increased the level of spontaneous chromosome aberrations in SLF2/SMC5

441 mutant cells lines complemented with an empty vector but not with WT SLF2 or SMC5 (Supplementary

442 Figure 19c).

443   It is known that the SMC5/6 complex is important for the dissolution of replication stress-

444 induced recombination, especially at repetitive regions prone to forming secondary structures and

445 natural replication pause site intermediates[41,43-46]. This is consistent with our observations that the

446 replication stress phenotype observed in SLF2/SMC5 mutant cells was not markedly exacerbated by

447 exposure to MMC, APH and HU (Figure 5; Supplementary Figures 11 and 13). Recently, it has been

448 shown that RNF168, which promotes the recruitment of the RAD18-SLF1/2-SMC5/6 pathway to

449 damaged replication forks, is important for signalling the presence of G-quadruplex (G4) DNA structures

450 stabilised by the RNA polymerase I inhibitor, CX5461[47]. Since cells deficient in BRCA1/2 and the

451 cohesin-associated helicase DDX11 are also hypersensitive to this agent[48,49] and DDX11 was shown

452 to function with SMC5/6 to repair DNA damage[17,50,51], we hypothesised that the RAD18-SLF1/2-

453 SMC5/6 pathway might play a role in suppressing replication stress at sites of stabilised G4 structures.

454 To test this possibility, we first investigated the effects of CX5461 on DNA replication using DNA fibre

455 analysis. This revealed that whilst WT SLF2 and SMC5 expressing patient fibroblasts could replicate

456 normally in the presence of CX5461, SLF2 and SMC5 patient fibroblasts complemented with an empty

457 vector exhibited a significant reduction in replication fork speed when incubated with this G4-stabilizing

458 compound (Figure 8d). Additionally, SLF2 and SMC5 patient-derived fibroblasts, LCLs and U-2 OS

459 SLF2 HM cells treated with CX5461 exhibited increased levels of G1 phase 53BP1 bodies and

460 chromosome aberrations (Figure 8e, Supplementary Figure 20a, c). In keeping with this, LCLs from

461 SLF2-P1 and SMC5-P8 displayed an increased sensitivity to CX5461 (Figure 8f). Strikingly, we also

462 observed that CX5461 treatment induced a significant increase in the levels of type 1 segmented

463 chromosomes in the SLF2 and SMC5 patient LCLs, but not in the WT LCLs (Supplementary Figure

464 20b). These data suggest a role for SLF2 and the SMC5/6 complex in resolving replication stress at

465 sites of stabilised G4 structures.

466   Whilst CX5461 is known to inhibit RNA polymerase I and stabilise G-quadruplexes, more

467 recently it has also been identified as a TOP2 poison[52,53]. Given the pleiotropic nature of CX5461, we

468 sought to identify which genotoxic lesion induced by CX5461 was causing the increased replication

469 stress in cells deficient in components of the SMC5/6 complex. In this respect, we carried out DNA fibre

470 and chromosomal aberration analysis on patient-derived cell lines following exposure to pyridostatin (a

471   G-quadruplex stabiliser), etoposide (a TOP2 poison) and BMH21 (an RNA polymerase I inhibitor).

472   Interestingly, only exposure to pyridostatin caused a significant reduction in replication progression and

473   an increase in the levels of chromosome aberrations in SLF2 and SMC5 mutant cell lines (Figure 8g,

474   Supplementary Figure 20d).

475   Taken together, these observations support the notion that the spontaneous replication stress

476   and chromosomal instability displayed by cells from patients with SLF2/SMC5 mutations is caused, in

477   part, by an inability to resolve a specific subset of replication-associated recombination intermediates

478   arising at sites of G4 structures.

479

Discussion

480

481   Disrupting the delicate balance between stem cell proliferation and differentiation profoundly

482 affects embryonic development, particularly body growth and brain development. Rapidly proliferating

483 pluripotent stem cells exhibit constitutively high levels of replication stress and as such are heavily

484 reliant on replication-associated DNA damage response pathways to maintain genome stability.

485 Unsurprisingly, patients with pathogenic variants in genes encoding components of the replisome, the

486 DNA damage response (DDR) and factors that maintain sister chromatid cohesion exhibit

487 developmental abnormalities including severe microcephaly and dwarfism. Furthermore, variants in

488 centrosome components and regulators of the microtubule-spindle network can also result in these

489 developmental abnormalities by affecting the orientation of the spindle pole and/or triggering excessive

490 cell death through the generation of aneuploid cells[1]. However, it is often difficult to determine whether

491 the cellular pathology underlying the development of these neurodevelopmental disorders results

492 primarily from the presence of aberrant replication or defective mitosis[38,54,55].

493   Here we report the clinical and genetic characterization of 11 patients with biallelic variants in

494 two components of the newly described RAD18-SLF1/2-SMC5/6 DDR pathway, *SLF2* and *SMC5*,

495 exhibiting microcephaly, short stature, cardiac defects and anaemia. However, in contrast to FA and

496 other known disorders, cells from these patients exhibit a unique chromosomal instability phenotype,

497 hallmarked by segmented and dicentric chromosomes and mosaic variegated hyperploidy, arising from

498 a combination of replication stress- and mitosis-associated cellular pathologies. Given that the

499 segmented chromosomes seen in SLF2 and SMC5 patient cells represent a chromosome instability

500 phenotype not previously associated with any known DNA repair or replication deficiency disorder, we

501 have named this syndrome, Atelís Syndrome (ATS), after the Greek word for incomplete to signify the

502 importance of these atelic or segmented chromosomes as a diagnostic marker of the disease.

503   The SMC5/6 complex has been shown to have many functions in the cell, including regulating

504 homologous recombination (HR)-dependent DNA repair, stabilising and restarting stalled replication

505 forks, maintaining replication through highly repetitive regions of the genome, maintaining rDNA

506 stability, elongating telomeres by ALT and controlling the topology of unusual DNA structures[12,14,56,57].

507 In contrast, little is known about the functions of SLF1 and SLF2, which were identified during a large

508 proteomic screen of proteins associated with damaged replication forks[11]. However, it has been

509 suggested that SLF1 and SLF2 are functional orthologs of the yeast Nse5 and Nse6 proteins,

510   respectively, which are important for localising the SMC5/6 complex to DNA damage and regulating its

511   ATPase activity[11,58-60].

512   Pursuant to the role of the SLF1/2-SMC5/6 complex in maintaining replication fork stability, we

513   demonstrate that cells from ATS patients exhibit elevated levels of spontaneous replication stress,

514   although this was not exacerbated significantly following exposure to replication stress-inducing agents

515   (HU, MMC or APH). This suggests that the clinical phenotype resulting from variants in *SLF2* and *SMC5*

516   may not simply arise from elevated levels of replication stress, but rather from deficits with a subset of

517   replication forks, such as those replicating through difficult-to-replicate regions of the genome or

518   encountering specific types of endogenous DNA lesions. Consistent with this hypothesis, ATS cells fail

519   to replicate efficiently in the presence of stabilised G4 structures and accumulate chromosomal

520   damage, suggesting that the RAD18-SLF1/2-SMC5/6 pathway functions to resolve replication

521   intermediates occurring at these lesions. Since G4 structures have been shown to be enriched at

522   telomere repeat sequences[61], a defect in the ability to replicate through these lesions could result in

523   genome instability at telomeres, potentially explaining the presence of dicentric chromosomes in ATS

524   patient cells.

525   ATS patients exhibit overlapping clinical and cellular features with WABS patients, including

526   microcephaly, growth restriction, skin hyper-pigmentation, ocular abnormalities and heart defects.

527   Moreover, cell lines derived from both ATS and WABS patients exhibit loss of sister chromatid cohesion

528   and premature chromatid separation[49]. Interestingly, the loss of sister chromatid cohesion in WABS cell

529   lines is exacerbated upon exposure to replication stress-inducing genotoxins, including G4 stabilising

530   agents[49]. Notably, cells from *Ddx11* null mice display loss of sister chromatid cohesion, chromosome

531   segregation errors and aneuploidy, which has been shown to induce a G2/M cell cycle delay and

532   apoptosis[62]. This suggests that a failure to resolve specific endogenous DNA lesions, such as G4

533   structures, in ATS cells may directly compromise cohesion, or exacerbate a pre-existing cohesion

534   defect, thus giving rise to chromosome segregation defects and aneuploidy that triggers cell death in

535   highly proliferative tissues, such as the developing brain.

536   It is clear that the RAD18-SLF1/2-SMC5/6 pathway plays additional cellular roles beyond

537   promoting replication through G4 lesions. In yeast, the smc5/6 complex restrains recombination at

538   programmed fork pause sites, for example, in the rDNA locus[43,44,63] and, in mammalian cells, SMC5/6

539   is involved in suppressing HR at highly repetitive sequences, e.g. rDNA, centromeres and

540 telomeres[14,63]. Consistent with this, ATS cells exhibit elevated levels of RAD51 in S-phase cells and

541 spontaneous SCEs and tSCEs. Interestingly, segmented chromosomes have been observed in cells

542 that have a combined defect in both the Holliday junction dissolution and resolution pathways[64],

543 indicating that the gaps in the type 1 segmented chromosomes may result from a failure to

544 dissolve/resolve recombination intermediates[41].

545   Cells from *NSMCE2* and *NSMCE3* mutant patients are not known to display segmented or

546 dicentric chromosomes, and whilst *NSMCE3* patient-derived cells exhibit aneuploidy and structural

547 chromosome abnormalities, hyperploidy to the extent seen in ATS cells was not reported[18,20]. This

548 indicates that neither NSMCE2 nor NSMCE3 subunits are essential for this SMC5/6 function, or that

549 the hypomorphic variants in these genes retain sufficient function to suppress these chromosomal

550 phenotypes. Consistent with the latter scenario, *Nsmce2* transgenic mice lacking SUMO E3 ligase

551 activity developed normally, whereas a complete loss of *Nsmce2* resulted in early embryonic lethality

552 associated with chromosome segregation defects[65]. Notably MEFs derived from the *Nsmce2* knockout

553 mice exhibited increased spontaneous replication stress and genome instability due to a failure to

554 detangle recombination intermediates similar to ATS patient cell lines (e.g. elevated levels of BRCA1

555 foci, increased sister chromatid and telomeric SCEs and chromosomal segregation errors)[65] indicating

556 that ATS represents a more severe form of SMC5/6 dysfunction.

557   Interestingly, the clinical phenotype exhibited by patients with variants in the SMC5/6 complex

558 components *NSMCE2* and *NSMCE3* are different from each other, with the former being associated

559 with microcephalic primordial dwarfism and insulin resistance[20] and the latter being associated with

560 severe pulmonary disease and immunodeficiency[18,19]. It is unclear why these clinical presentations are

561 different, especially as the cellular phenotype resulting from *NSMCE2* and *NSMCE3* variants are

562 similar[18,20]. One possible important cellular difference between the two disorders is that the patient-

563 associated missense variants in *NSMCE3* result in the destabilization of the SMC5/6 complex to a much

564 greater extent than the nonsense variants present in *NSMCE2* patients[18,20]. It is notable that the clinical

565 phenotype of ATS patients more closely resembles that of *NSMCE2* patients than *NSMCE3* patients,

566 and like *NSMCE2* patient variants, *SLF2* and *SMC5* patient variants do not destabilise the SMC5/6

567 complex to any significant degree.

568   Taken together, we have demonstrated that variants in two components of the RAD18-SLF1/2-

569 SMC5/6 pathway give rise to a FA/MVA-like disorder, termed Atelís Syndrome, with clinical and cellular

570   features overlapping with WABS, MVA, NSMCE2 variants and FA. In vivo ablation of *slf2* and *smc5* in

571   zebrafish recapitulate patient phenotypes including microcephaly and craniofacial patterning defects,

572   likely due to concomitant cell cycle defects and apoptosis. We show that cells from ATS patients display

573   a unique and complex chromosomal instability phenotype consisting of atelic (segmented) and dicentric

574   chromosomes coupled with MVH, which should allow for cytogenetic diagnosis of patients with this

575   disorder.

576

577   **Methods**

578

579   **Research subjects.**

580   Informed consent was obtained from all participating families to take clinical samples and to

581   publish clinical information in accordance with local approval regulations and in compliance with the

582   Declaration of Helsinki principles. This study was approved by the West Midlands, Coventry and

583   Warwickshire Research Ethics Committee (REC: 20/WM/0098), the Scottish Multicentre Research

584   Ethics Committee (REC: 05/MRE00/74), the Lancaster General Hospital Institutional Review Board and

585   the Institutional Review Boards of Yokohama City University Graduate School of Medicine (ID:

586   A190800001) and Jichi Medical University (ID: G21-V06). A collaboration to study the pathological

587   significance of the identified *SLF2* and *SMC5* variants was established via GeneMatcher[66].

588

589   **Exome sequencing.**

590   Genomic DNA from affected children and family members was extracted from peripheral blood

591   using standard methods. Whole exome capture and sequencing was performed as described to a

592   minimum of *30x* coverage[67]. Exome sequencing for families 8 and 9 was conducted in collaboration

593   with the Regeneron Genetics Center as previously described[68]. Briefly, DNA was sheared (Covaris S2),

594   exome capture performed using the Agilent SureSelect v5 enrichment kit according to manufacturer's

595   instructions, and libraries were sequenced with 125 bp read-pairs using the Illumina HiSeq 2500 V4

596   platform. All analyses were performed as described[69]. Variants were confirmed by bidirectional capillary

597   dye-terminator sequencing and annotated using the reference sequences, GenBank: NM_018121.4,

598   NM_001136123.2 and NM_015110.4. Capillary sequencing was performed in the MRC Human

599   Genetics Unit, Edinburgh, UK, the University of Birmingham, UK, the Bioscientia Institute for Medical

600   Diagnostics, Germany, the Rare Disease Genomics Department, Yokohama City University Hospital,

601   Japan and the Regeneron Genetics Center, Regeneron Pharmaceuticals Inc., USA.

602

603   **Cell lines**

604   Patient-derived lymphoblastoid cell lines (LCLs) were generated from peripheral blood samples

605   with Epstein Barr virus (EBV) transformation using standard methods and were maintained in

606   RPMI1640 medium (Life Technologies) supplemented with 10% FBS, L-glutamine and penicillin-

607   streptomycin. The ATR-Seckel LCL used in this study was reported previously[31]. Dermal primary

608   fibroblasts were grown from skin-punch biopsies and maintained in Dulbecco's modified Eagle's

609   medium (DMEM; Thermo Fisher Scientific) supplemented with 20% FCS, 5% L-glutamine and 5%

610   penicillin-streptomycin. Primary fibroblasts were immortalized with a lentivirus expressing human

611   telomerase reverse transcriptase (hTERT) that was generated by transfecting 293FT cells (Thermo

612   Fisher Scientific) with the plasmids: pLV-hTERT-IRES-hygro (Addgene #85140), psPax2 (Addgene

613   #12260) and pMD2.G (Addgene #12259). Selection was performed using Hygromycin (Thermo Fisher

614   Scientific) at 70 µg/ml. All LCLs were routinely grown in RPMI-1640 (Thermo Fisher Scientific)

615   supplemented with 10% FCS, 5% L-glutamine and 5% penicillin-streptomycin. Patient cell lines were

616   validated using Sanger sequencing and immunoblotting. Fibroblast and U-2 OS cell complementation

617   was carried out using the pLVX-IRES-Neo lentiviral vector (Takara Bio) encoding *2x*Myc-tagged *SLF2*

618   or untagged *SMC5*.

619   293FT (Thermo Fisher Scientific) were maintained in DMEM supplemented with 10% FBS, 5%

620   l-glutamine and 5% penicillin-streptomycin and U-2-OS cells were cultured in McCoy's 5A medium,

621   supplemented with 10% FBS, and 5% penicillin/streptomycin. 293FT transiently transfected with GFP-

622   BLM or GFP expression vectors using Lipofectamine 2000 (Thermo Fisher Scientific). U-2 OS cells

623   were transiently transfected with SLF2/SMC5 expression vectors using FuGENE 6 Transfection

624   Reagent (E2692, Promega) or Lipofectamine 3000 Reagent (L3000015, Thermo Fisher Scientific)

625   where indicated. Stable GFP-SMC5 cell lines were generated by G418 selection and low expressing

626   clones were selected based on GFP expression. All cell lines were routinely tested for mycoplasma.

627

628   **Western blotting**

629   Whole-cell extracts were obtained by sonication in UTB buffer (8 M urea, 50 mM Tris, 150 mM

630   β-mercaptoethanol) and analyzed by SDS–PAGE following standard procedures. Protein samples were

631   run on 6–12% acrylamide gels with SDS–PAGE and transferred onto a nitrocellulose membrane.

632   Immunoblotting was performed using antibodies to: RAD18 (Fortis Life Sciences, A301-340A; 1:1000),

633   SMC5 (Fortis Life Sciences, A300-236A; 1:500), SMC6 (Fortis Life Sciences, A300-237A; 1:2000),

634   SLF2 (generated in house; 1:1000)[11], GAPDH (Genetex, GTX100118; 1:1000), Myc (Abcam, ab32;

635   1:1000), GFP (SCBT, sc-9996), HA (SCBT, sc-7392), α-Tubulin (Sigma-Aldrich, T9026), ATR (Fortis

636   Life Sciences, A300-137A; 1:1,000), phospho-ATR (Thr1989) (GeneTex, GTX128145; 1:500),

637   FANCD2 (SCBT, sc-20022; 1:1,000), CHK1 (SCBT, sc-8408; 1:1,000), phospho-CHK1 (Ser345) (Cell

638   Signaling Technology, 2341; 1:100), NBS1 (Genetex, GTX70224; 1:10,000); phospho-NBS1 (Ser343)

639   (Abcam, 47272; 1:500); SMC1 (Fortis Life Sciences, A300-055A; 1:1,000); phospho-SMC1 (Ser966)

640   (Fortis Life Sciences, A300-050A; 1:1,000); HA (Abcam, Ab9110; 1:1000). Loading controls for all blots

641   were derived from re-probing the same membrane, except for the phospho-antibody immunoblots, for

642   which paired gels were run simultaneously and blotted in parallel for phosphorylated and total proteins.

643

644   **Co-immunoprecipitation and GFP-Trap pull-downs**

645   For GFP-Trap pulldown experiments with 293FT cells, cells transfected with plasmids using

646   Lipofectamine 2000, were treated with 2 mM HU for 16 h and harvested. Cells were incubated in lysis

647   buffer (150 mM NaCl, 50 mM Tris HCl pH 7.5, 2 mM MgCl$_2$, 1% NP40, 90 U/ml Benzonase (Novagen)

648   and EDTA-free protease inhibitor cocktail [Roche]) for 30 min with rotation at 4 °C. Cell lysates were

649   then pre-cleared at 65,000 g at 4 °C for 30 min. For GFP-Trap, 3-5 mg of lysate was incubated with

650   GFP-Trap agarose beads (ChromoTek) for 5 h at 4 °C. The resulting GFP-Trap complexes were

651   washed with wash buffer (150 mM NaCl, 50 mM Tris HCl pH 7.5, 0.5% NP40, and complete protease

652   inhibitor cocktail [Roche]) and analysed by SDS–PAGE.

653   For immunoprecipitations from patient-derived LCLs, 3 mg of lysate (prepared with the same

654   lysis buffer as above) was immunoprecipitated with 5 µg of antibody (RAD18; Fortis Life Sciences,

655   A301-340A or NSMCE2; Fortis Life Sciences, A304-129A) and protein A-sepharose beads (GE

656   Healthcare). Complexes were washed with wash buffer (as described above) and analysed by SDS-

657   PAGE. Experiments were carried out in the presence of Benzonase nuclease to exclude the possibility

658   of interactions being mediated by DNA.

659   For immunoprecipitations from U-2 OS cells, cell lysates were generated using EBC buffer

660   (150mM NaCl; 50mM Tris, pH 7.5; 1mM EDTA; 0.5% IGEPAL CA-630). Lysates were subject to Co-IP

661   using Strep-Tactin Sepharose (IBA GmbH) prior to immunoblot using the following antibodies: GFP (sc-

662   9996, SCBT), HA (sc-7392, SCBT), RAD18 (A301-340A, Fortis Life Sciences), SMC6 (A300-237A,

663   Fortis Life Sciences), SMC5 (Fortis Life Sciences, A300-236A), NSMCE2 (Fortis Life Sciences, A304-

664   129A), α-Tubulin (T9026, Sigma-Aldrich).

665

666   **Laser micro-irradiation**

667   U-2 OS cells were grown on coverslips and sensitized to laser induced DSB formation using 5-

668   Bromo-2-deoxyuridine (B9285-50MG, Sigma-Aldrich) for 24 h. GFP-SLF2 expression vectors were

669   transiently transfected 24 h prior and GFP-SMC5 stable expressing cells were used for micro-

670   irradiation. Laser micro-irradiation induced DSB formation was performed as previously described[70]

671   with 1 h allowed for recovery. Cells were pre-extracted using CSK buffer (100 mM NaCl, 10 mM HEPES,

672   3 mM $MgCl_2$, 300 mM Sucrose, 0.25% Triton-X100, 1 mM PMSF) prior to fixation in formalin buffer

673   (AMPQ43182, VWR) for 15 mins at room temperature (RT).

674   Fixed coverslips were blocked with 5% Bovine Serum Albumin (A7906, Sigma-Aldrich) for 1 h

675   prior to staining with anti-γ-H2AX (Ser139) (1:1000, 05-636, Merck) and anti-GFP (1:500, PABG1,

676   Chromotek) overnight at 4 °C. After PBS washes cells were stained with Alexa Fluor secondary

677   antibodies and 4',6-Diamidino-2-Phenylindole (DAPI, D1306, Molecular Probes) for 30 mins at RT. After

678   further washing, coverslips were dried completely and mounted for imaging using Mowiol (81381,

679   Sigma-Aldrich).

680

681   **Zebrafish husbandry and embryo maintenance.**

682   All zebrafish experiments were performed according to protocols approved by the Duke

683   University and Northwestern University institutional animal care and use committees (IACUC). Wild

684   type (WT: ZDR or NIH) adults or transgenic *-1.4col1a1:egfp*[25] adults were maintained on an AB

685   background and subjected to natural matings to generate embryos for microinjection and/or

686   phenotyping. Embryos were grown in egg water (0.3 g/L NaCl, 75 mg/L $CaSO_4$, 37.5 mg/L $NaHCO_3$,

687   0.003% methylene blue) at 28 °C until assessment. Zebrafish sex is unknown until animals are ~3

688   months old. Therefore, in the larvae at <5days post fertilization, it is not possible to know how many

689   males and females are present, and there should be no sex-dependent effects at this stage. However,

690   adults that were used to generate embryos were crossed in a 1 male to 1 female ratio.

691

692   **CRISPR-Cas9 genome editing of zebrafish embryos**

693   Reciprocal translated BLAST of human *SLF2* (NP_060591.3) and *SMC5* (NP_055925.2) was

694   performed against the zebrafish genome and found a single ortholog corresponding to either protein

695   (transcripts targeted: *slf2*: ENSDART00000136689.3, *smc5*: ENSDART00000122170.4). To identify

696   CRISPR/Cas9 single guide RNA (sgRNA) targets in both genes, CHOPCHOPv2[71] (and

697   http://chopchop.cbu.uib.no) was used. sgRNAs were generated using the GeneArt precision gRNA

698   synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions (Supplementary

699   Table S11). 1 nl of cocktail containing 100 pg sgRNA with or without 200 pg of Cas9 protein (PNA Bio)

700   was injected into the cell of single cell staged zebrafish embryos. To estimate the percentage mosaicism

701   of genome-edited cells, genomic DNA from individual embryos was extracted at 2 days post fertilization

702   (dpf; two controls and ten founder [F0] embryos per sgRNA). PCR was used to amplify the sgRNA

703   targeted region using flanking primers and heteroduplex analysis was performed using polyacrylamide

704   gel electrophoresis (PAGE). PCR products were denatured, reannealed slowly, and migrated on a 20%

705   polyacrylamide gel (Thermo Fisher Scientific). PCR products from five embryos per sgRNA were

706   randomly selected from the heteroduplex analysis, cloned into a TOPO-TA vector (Thermo Fisher

707   Scientific) and sequenced using BigDye terminator 3.1 chemistry (Applied Biosystems). To isolate

708   stable *slf2* mutants, F0 animals were crossed to WT ZDR adults and heterozygous F1 mutants bearing

709   the c.515_522del (p.Ser172_Ser174fs191Ter) variant were identified. Mutant F1 adult siblings were

710   inter-crossed to generate homozygous F2 animals for phenotyping. *slf2* mRNA expression level was

711   monitored by qRT-PCR (QuantStudio, Thermo Fisher Scientific) using SYBR Green detection kit

712   (Thermo Fisher Scientific) with normalization to β-*actin*.

713

714   **Transient suppression of *slf2* and *smc5* in zebrafish embryos**

715   Splice blocking morpholinos (MOs) were designed to target the *slf2* exon 11 (e11i11) and *smc5*

716   exon 3 (e3i3*)* splice donor sites (Gene Tools; Supplementary Table S11)). Each gene was transiently

717   suppressed independently by injecting 1 nl at different doses (3 ng, 6 ng and 9 ng) into one to four cell

718   staged zebrafish embryos. To validate MO efficiency, total RNA was extracted from pools of 2 dpf

719   embryos (25 animals/condition; controls and MO-injected) using Trizol (Thermo Fisher Scientific)

720   according to manufacturer's instructions. cDNA was synthesised with the QuantiTect Reverse

721   Transcription kit (Qiagen), RT-PCR of the MO target locus was performed, and PCR products were

722   separated on a 1% agarose gel. Resulting PCR bands were gel purified with the QIAquick gel extraction

723   kit (Qiagen) and cloned into the TOPO-TA cloning vector (Thermo Fisher Scientific). Purified plasmids

724   from resulting colonies (n=4/PCR product) were sequenced using BigDye 3.1 terminator chemistry

725   according to standard protocols.

726

Molecular cloning and site directed mutagenesis of human *SLF2* and *SMC5* constructs for expression of human proteins in zebrafish

Full length Gateway-compatible *SLF2* (NM_018121.4) and *SMC5* (NM_015110.4) open reading frame (ORF) entry vectors were obtained (Horizon). WT ORFs of both genes were inserted into a pCS2+ Gateway destination vector using LR clonase II (Thermo Fisher Scientific). *SMC5* variants identified in either affected individuals (p.His990Asp, p.Arg372del, p.Arg425Ter) or in gnomAD (dbSNP ID: rs59648118, p.(Arg733Gln); 16 homozygotes of 140,814 individuals, negative control) were inserted using site directed mutagenesis as described (Supplementary Table S11)[72]. After full ORF sequence confirmation of all WT and mutant plasmids, each construct was linearised with NotI and in vitro transcription was performed with the mMessage mMachine SP6 Transcription kit (Thermo Fisher Scientific) according to manufacturer's instructions. 150 pg *SLF2* mRNA with 6 ng *slf2* MO and 150 pg *SMC5* mRNA with 9 ng *smc5* MO was used for in vivo complementation assays.

Live imaging of zebrafish larvae

Images of tricaine-anesthetized larvae at 3 dpf were captured using the Vertebrate Automated Screening Technology (VAST) Bioimager (Union Biometrica) mounted to an AXIO Imager.M2m microscope (Zeiss) with a *10x* objective lens. Larvae were passed sequentially through a 600 μm capillary on the detection platform. Each larva was detected by software on the computer screen and oriented automatically for lateral and ventral side images with a pre-provided template setting in the software. VAST software (version 1.2.6.7) operated in automatic imaging mode with a 70% minimum similarity threshold, as described[73]. Bright field lateral images were captured with the VAST onboard camera and a fluorescent signal from ventrally positioned larvae with an Axiocam 503 monochrome camera (Zeiss) and ZenPro software (Zeiss).

TUNEL assay and phospho-histone H3 (pHH3) immunostaining in zebrafish larvae

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assays or pHH3 immunostaining on whole mount embryos were performed as described[27,74,75]. Embryos were dechorionated at 2 dpf (*slf2* and *smc5*) or 3 dpf (*smc5*) and fixed overnight in 4% paraformaldehyde (PFA) at 4 °C. Embryos were then dehydrated in methanol at -20 °C for 2 h and gradually rehydrated in methanol in PBS and 0.1% Tween (PBST) in the following percent volume/volume ratios: 75/25;

757 50/50; 25/75 for 10 min each at RT. Embryos were bleached for 12 min in a solution of 9 ml PBST + 1

758 ml H_2O_2 + 0.05 g KOH before proteinase K treatment and fixation in 4% PFA for 20 min at RT. For

759 TUNEL, embryos were then incubated in equilibration buffer for 1 h and treated overnight with TdT

760 enzyme at 37 °C in a humidified incubator. Following treatment with digoxigenin (ApopTag red in situ

761 apoptosis detection kit, Sigma-Aldrich) for 2 h, embryos were washed *3x* with PBST (10 min each) and

762 processed for imaging. For pHH3 staining, embryos were washed *3x* (10 min each) with PBST and

763 incubated in blocking solution (IF buffer [1% BSA in PBST] +10% FBS]) for 1 h. Embryos were then

764 treated with primary antibody diluted in 1% BSA overnight: anti-pHH3 (SCBT, sc-374669: 1:500) at 4

765 °C. Following staining with a secondary antibody: Alexa Fluor 488 goat anti-rabbit IgG (Thermo Fisher

766 Scientific, A11008: 1:500) diluted in 1% BSA for 2 h at RT, embryos were washed *2x* (10 min each)

767 with IF buffer and processed for imaging. For both TUNEL and pHH3 stained embryos, a z-stacked

768 fluorescent signal of the dorsal aspect was captured with a Nikon AZ100 microscope facilitated by a

769 Nikon camera controlled by Nikon NIS Elements Software.

770

771 **Zebrafish image analysis**

772 ImageJ (NIH) was used to measure lateral head size, ceratohyal angle and count cells (TUNEL

773 or pHH3) in the specified head region. Raw images were exported as TIF files and contrast and

774 brightness were adjusted using identical settings for all images across the experiments. To measure

775 head size, a straight line was drawn from the posterior otolith to the tip of the mouth (line a), the dorsal

776 head area outlined (line b), and the arbitrary shape closed with a line perpendicular to line a (line c).

777 Ceratohyal angle was measured with the angle tool. To count TUNEL or pHH3 positive cells, the image-

778 based tool for counting nuclei (ICTN) plugin for ImageJ was used. A consistent region between the two

779 eyes was selected that spanned the most anterior region of the head to the most anterior region of the

780 yolk.

781

782 **Immunofluorescence in human cells**

783 Patient-derived fibroblasts or U-2 OS CRISPR HM cells were seeded onto coverslips at least

784 48 h before extraction and fixation. Cells were pre-extracted for 5 min on ice with ice-cold extraction

785 buffer (25 mM HEPES [pH 7.4], 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 300 mM sucrose, and 0.5%

786 Triton X-100) and then fixed with 4% paraformaldehyde (PFA) for 10 min. For immunofluorescence

787   involving patient-derived LCLs, cells were seeded onto Poly-L-Lysine coated coverslips 20 min before

788   fixation with ice-cold methanol for 20 min. For immunofluorescence using cells treated with exogenous

789   DNA damage, patient-derived fibroblasts or LCLs cells were incubated with 500 nM APH, 50 ng/ml

790   MMC or 250 µM CX5461 (Selleck Chemicals, S2684), as indicated in the figure legends, 24 h before

791   fixation.

792   Fixed cells were then stained with primary antibodies specific to γH2AX (Sigma-Aldrich, 05-

793   636; 1:1,000), CENPA (Abcam, Ab13939; 1:750), 53BP1 (Novus Biologicals, NB100-304; 1:1,000),

794   CENPF/Mitosin (Abcam, Ab5; 1:500 and BD Transduction Laboratories, 610768; 1:500), α-Tubulin

795   (Sigma-Aldrich, B-5-1-2; 1:4000), PCNT (Abcam, Ab4448; 1:100), and RAD51 (Merck, PC130; 1:500),

796   and with secondary antibodies: anti-rabbit IgG Alexa Fluor 488 (Thermo Fisher Scientific, A11070;

797   1:1000) and anti-mouse IgG Alexa Fluor 594 (Thermo Fisher Scientific, A11032; 1:1000). Cells were

798   then stained with DAPI and visualized with a *100x* oil-immersion objective lens on a Nikon Eclipse Ni

799   microscope.

800   To visualize DNA replication, cells were incubated in medium containing 10 µM EdU for 30-45

801   min before harvesting. EdU immunolabeling was performed using the Click-iT EdU Imaging Kit (Thermo

802   Fisher Scientific, C10337) according to the manufacturer's protocol.

803

804   **DNA fibre spreading assay**

805   Patient-derived fibroblasts or U-2 OS cells were seeded at least 48 h prior to harvesting. Cells

806   were incubated with 25 mM CldU for 30 min, washed with media containing 250 mM IdU (with or without

807   250µM CX5461), incubated with 250 mM IdU (with or without 250 µM CX5461) for 30 min, and

808   harvested by trypsinization. For patient-derived LCLs, untreated cells were incubated with 25 mM CldU

809   for 20 min, washed with media containing 250 mM IdU, before being incubated with 250 mM IdU for 20

810   min and harvested. LCLs were incubated with 50 ng/ml MMC for 24 h prior to pulse labelling with 25

811   mM CldU for 20 min and then 250 mM IdU for 20 min. For all incubation or washing steps, 50 ng/ml

812   MMC was present in the media. For cells treated with HU, after being incubated with 25 mM CldU for

813   20 min, LCLs were incubated with media containing 2 mM HU for 2 h, before being washed in media

814   containing 250 mM IdU, then incubated with 250 mM IdU for 20 min and harvested.

815   Following harvesting, cells were washed with PBS and resuspended to a concentration of

816   500,000 cells/ml in PBS, and then lysed in lysis buffer (200 mM Tris-HCl [pH 7.5], 50 mM EDTA, 0.5%

817 SDS) directly on glass microscope slides. DNA fibres were spread down the slide by gravity, fixed in

818 methanol/acetic acid (3:1) and denatured with 2.5 M HCl. The thymidine analogues, CldU and IdU,

819 were detected via rat anti-BrdU antibody (clone BU1/75, ICR1; Abcam, ab6326; 1:500) and mouse anti-

820 BrdU antibody (clone B44; BD Biosciences, 347583; 1:500) respectively, and secondary antibodies

821 conjugated to Alexa Fluor 594 or Alexa Fluor 488 (Thermo Fisher Scientific). Labelled DNA fibres were

822 visualized with a Nikon Eclipse Ni microscope with *100x* oil-immersion objective lenses, and images

823 were acquired with NIS-Elements software (Nikon Instruments). Replication fork structures and CldU

824 and IdU track lengths were then quantified with ImageJ software (US NIH).

825

826 **Metaphase spreads**

827 Giemsa-stained metaphase spreads from patient-derived cell lines or U-2 OS CRISPR SLF2

828 HM cells were prepared by adding of 0.2 mg/ml colcemid (KaryoMAX, Life Technologies) and

829 incubating for 3 h. The cells were then harvested by trypsinization, subjected to hypotonic shock for 30

830 min at 37 °C in hypotonic buffer (10 mM KCl, 15% FCS), and fixed in ethanol/acetic acid solution (3:1).

831 The cells were dropped onto microscope slides, stained for 15 min in Giemsa-modified solution (Sigma-

832 Aldrich; 5% vol/vol in water), and washed in water for 5 min. For analysis of cohesion fatigue in SLF2

833 patient LCLs, the metaphase spread protocol was followed as above. However, instead of adding

834 colcemid, 25µM MG132 (Sigma-Aldrich, M7449) was added 4 h before harvesting.

835 To prepare Giemsa-stained metaphase spreads from peripheral blood, whole blood was diluted

836 in RPMI1640 and 180 µg/ml PHA (Thermo Fisher Scientific) was added for 48-72 h at 37 °C. 4 h prior

837 to harvesting 0.2 mg/ml colcemid was added. The cells were pelleted and then subjected to hypotonic

838 shock for 10 min at 37 °C in hypotonic buffer (0.075M KCl). Finally, the cells were then fixed in

839 methanol/acetic acid solution (3:1) and processed as described above.

840

841 **Fluorescence in situ hybridization**

842 For Fluorescence In Situ Hybridization (FISH) was carried out on peripheral blood lymphocytes

843 metaphases using a peptide nucleic acid (PNA) pan-centromere FISH probe conjugated to Alexa Fluor

844 488 (5'-ATTCGTTGGAAACGGGA-3', PNA Bio, F3004 CENPB-Alexa488). Briefly, the PNA FISH

845 probes was made up as per the manufacturer's instructions. Metaphase spreads were harvested from

846 patient blood samples as above, and metaphases were dropped onto acetic-acid humidified microscope

847 slides. 24 h later, the slides were re-hydrated in PBS, dehydrated in an ethanol series (70%, 95%,

848 100%) and air dried. The slides were pre-warmed to 37 °C and before being incubated with hybridization

849 buffer (20 mM Tris, pH7.4, 60% formamide, 0.5% blocking reagent [Roche Blocking Reagent,

850 11096176001], 1% v/v PNA probe) for 10 min at 85 °C. The slides were then incubated in a dark,

851 humidified chamber at RT for 2 h, before being washed in wash buffer (70% formamide, 10mM Tris)

852 and dehydrated in an ethanol series (70%, 95%, 100%). The slides were then air dried and fixed with

853 prolong gold DAPI mounting medium (ProLong Gold Antifade Mountant with DAPI, P36935).

854

855 **Sister chromatid exchange analysis**

856 For sister chromatid exchange analysis, LCLs were incubated with 10 µM BrdU for 48 h before

857 incubating with 0.2 µg/ml demecolcine for 3 h. Cells were then resuspended in 0.075M KCl and

858 incubated at 37 °C for 1 h, fixed in methanol/acetic acid (3:1) and dropped onto microscope slides. The

859 slides were then incubated in 10 µg/ml Hoescht for 20 min and exposed to UVA light for 1 h in 2× SSC

860 buffer. Slides were incubated in 2× SSC buffer for 1 h at 60 °C and stained with 5% Giemsa. For

861 metaphase spread analysis of cells treated with exogenous DNA damage, patient-derived LCLs cells

862 were incubated with 500 nM APH or 50 ng/ml MMC 24 h before harvesting.

863 For analyses of telomere sister chromatid exchange, LCLs were cultured in the presence of

864 BrdU:BrdC (final concentration of 7.5 mM BrdU [MP Biomedicals, 100166] and 2.5 mM BrdC [Sigma-

865 Aldrich, B5002]) for 10 hr prior to harvesting. KaryoMAX colcemid (Gibco, 15212-012) was added at a

866 concentration of 0.1 µg/mL during the last 2 h. Cells were collected and washed in 75 mM KCl. Cells

867 were then fixed *3x* in methanol:acetic acid (3:1) by adding fixative solution dropwise with constant gentle

868 agitation by vortex. Following fixation, cells were dropped onto microscope slides and metaphase

869 spreads were allowed to dry overnight. Next, slides were rehydrated in *1x* PBS and then treated with

870 0.5 mg/ml RNase A (Sigma-Aldrich, R5125) for 30 min at 37 °C. Next, slides were treated with 0.5 µg/ml

871 Hoescht 33258 (Sigma-Aldrich, 861405) in *2x* SSC for 15 min at RT, UV-irradiated, and digested with

872 ExoIII (NEB M0206L) for at least 30 min at 37 °C. Slides were then washed once in *1x* PBS and

873 dehydrated in an ethanol series (70%, 90%, 100%) and air dried. FISH was performed using a TelC-

874 Alexa488-conjugated PNA probe (PNA Bio, F1004; 1:1,000) followed by a TelG-Cy3-conjugated PNA

875 probe (PNA Bio, F1006; 1:1,000) diluted in hybridization solution (10 mM Tris-HCl pH 7.2; 70%

876 formamide; 0.5% blocking reagent (Roche, 11096176001)) each for 2 h at RT. Next, slides were

877   washed at RT twice for 30 min in PNA wash A (70% formamide, 0.1% BSA, 10 mM Tris pH 7.2) and *3x*

878   for 5 min in PNA wash B (100 mM Tris pH 7.2, 150 mM NaCl, 0.1% Tween-20). The second PNA wash

879   B contained DAPI (Life Technologies, D1306) at a 1:1000 concentration. Slides were then dehydrated

880   and dried as described above prior to mounting with Vectashield (Vectalabs, H1000). Slides were

881   imaged using a Zeiss Spinning Disk confocal microscope. Image analyses were blinded and used FIJI

882   version 2.1.0/153.c. Statistical analysis was performed using GraphPad Prism version 9.4.1.

883

884   **LCL proliferation assays**

885   LCL proliferation assays were carried out as previously reported[49]. Briefly, LCLs were seeded at a

886   concentration of 0.25×10^6 cells per ml in 25 cm^2 flasks and incubated with an increasing concentration

887   of CX5461. The treated cells were counted when the untreated cells had reached a concentration of

888   2.0×10^6 cells per ml (approximately three population doubling times). The viability of the cells was

889   expressed as a percentage of the untreated cell count.

890

891   **Plasmids, mutagenesis and sequencing primers**

892   Total RNA was extracted from cell lines using RNeasy Mini kit (Qiagen) according to the

893   manufacturer's instructions. DNA was removed by treatment with DNase I (Qiagen), and cDNA was

894   generated using Superscript II and primed with oligo-dT (Thermo Fisher Scientific). PCR was carried

895   out using Phusion Hot Start II (Thermo Fisher Scientific). *2x*Myc-SLF2 or untagged SMC5 lentiviral

896   expression constructs were generated by cloning a PCR-generated cDNA into the NotI site of pLVX-

897   IRES-neo (Takara Bio). The SLF2 and SMC5 ORFs were verified by sequencing using the primers in

898   Supplementary Table S12.

899   Full length SLF2 cDNA was also cloned into pcDNA4/TO (Thermo Fisher Scientific) and

900   deletion constructs were generated using KOD Hot Start DNA polymerase (Merck) according to

901   manufacturer's instructions. The following primer sets in Supplementary Table S13 were used to

902   generate the SLF2 deletion constructs and SLF2 'minimal binding region' (MBR) constructs. GFP-SLF2

903   is previously described[11]. Full length SMC5 cDNA was amplified and cloned into pEGFP-C1 (Takara

904   Bio) using KpnI/BamHI. SLF2/SMC5 mutagenesis was achieved using the Q5 Site-Directed

905   Mutagenesis Kit (E0554S, NEB) according to manufacturer's instructions. The following primer sets in

906 Supplementary Table S13 were used to generate mutant expression vectors. SLF2 p.Gln1162His

907 variant was generated using gene synthesis (Thermo Fisher Scientific).

908 Lentiviral plasmids encoding the bacterial Holliday junction resolvase RusA were a kind gift

909 from Agata Smorgorzewska[40].

910

RT–PCR analysis of patient cells

912 RT-PCR of SLF2 was performed using transcript specific primers (Supplementary Table S14)

913 to assess the mRNA levels of the two longest annotated *SLF2* transcripts (NM_018121.4 and

914 NM_001136123.2) in patient whole blood RNA (Paxgene) or commercially-obtained human cDNA

915 panels: Human Universal QUICK-Clone II (Clontech), which is pool of cDNA obtained from 35 different

916 healthy adult or fetal tissues; and Human multiple tissue cDNA (MTC) panel I (Clontech). PCR product

917 was migrated on a 1% agarose gel for 40 minutes at 100V.

918

CRISPR-Cas9 genome editing of U-2 OS cells

920 Pairs of SLF2 targeting guide RNAs (sgRNA 1, 5'-AGTTTCATCACTCGGTTCCT-3'; sgRNA 2,

921 5'-GGCTTGGCACCTTCAAATTC-3') were designed using the CHOPCHOP web tool (version 2)[71,76]

922 and hybridised and ligated into the purpose built AIO-GFP All-in-One Cas9D10A nickase vector at

923 unique BbsI and BsaI sites. These constructs were transfected into U-2 OS cells using FuGENE

924 transfection reagent according to manufacturer's instructions (3:1 ratio of FuGENE to DNA). Cells were

925 sorted for high GFP expression by fluorescence-activated cell sorting (FACS) into 96-well dishes and

926 recovered in McCoys 5A media supplemented with 20% FBS and 5% penicillin-streptomycin. After

927 three weeks, 25 colonies were chosen to be propagated and screened for successful gene editing. After

928 propagating, potential clones were lysed in lysis butter (100 mM Tris/HCl pH 8.5, 5 mM EDTA, 0.2%

929 SDS, 200 mM NaCl, 100 μg Proteinase K/ml) and the DNA was precipitated with isopropanol and

930 resuspended in 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5. Screening of genomic DNA from clones was

931 achieved by sequencing a region of SLF2 surrounding the Cas9 nickase cut sites (Reverse primer, 5'-

932 AGTTCCGATAATCCACCCCTT-3'; Forward primer, 5'-TTTCTGCAACCAGGTAGTCCT-3'). Following

933 secondary screening of five clones by Western blotting, two SLF2 CRISPR HM clones were chosen

934 (renamed as cl.1 and cl.2) and were characterised further by inserting the amplified region of SLF2

935 described above into TOPO-TA vectors. 20 TOPO-TA vector clones were then sequenced for both cl.1

936   and cl.2 to identify all SLF2 mutant alleles and ensure no WT allele was present. The HM clones cl.1

937   and cl.2 were then complemented by 2xMyc tagged SLF2 cloned into pLVX-IRES-neo (Takara Bio).

938

939   **Statistical Analysis**

940   Statistical analyses were performed as indicated in the figure legends. A p-value of less than

941   0.05 indicates significance. The number of independent experimental replicates is denoted in the figure

942   legends. In all cases, independent experiments represent distinct samples, and not the same sample

943   measured repeatedly.

944

945

946

947 **Data Availability**

948 The datasets generated during WES are not publicly available due to reasons of sensitivity, e.g. human

949 data, but may be available from the corresponding author upon request subject to parental consent.

950 Gene variant frequency was obtained from the gnomAD database (https://gnomad.broadinstitute.org/).

951 Accession codes for genes/proteins analysed within this study are: Human SLF2 (NM_018121.4

952 [https://www.ncbi.nlm.nih.gov/nuccore/NM_018121.4], NM_001136123.2

953 [https://www.ncbi.nlm.nih.gov/nuccore/NM_001136123.2], NP_060591.3

954 [https://www.ncbi.nlm.nih.gov/protein/NP_060591.3]), Human SMC5 (NM_015110.4

955 [https://www.ncbi.nlm.nih.gov/nuccore/NM_015110.4], NP_055925.2

956 [https://www.ncbi.nlm.nih.gov/protein/NP_055925.2]), zebrafish slf2 (XM_002664123.6

957 [https://www.ncbi.nlm.nih.gov/nuccore/XM_002664123.6], XP_002664169.3

958 [https://www.ncbi.nlm.nih.gov/protein/XP_002664169.3]), zebrafish smc5 (NM_001193541.1

959 [https://www.ncbi.nlm.nih.gov/nuccore/NM_001193541.1], NP_001180470.1

960 [https://www.ncbi.nlm.nih.gov/protein/NP_001180470.1]). Plasmids obtained from Addgene

961 (https://www.addgene.org/) used in this study: pLV-hTERT-IRES-hygro (Addgene #85140), psPax2

962 (Addgene #12260) and pMD2.G (Addgene #12259). PDB files used within this study to model the

963 structural impact of SMC5 patient variants: Saccharomyces cerevisiae Smc5 (PDB: 3HTK), Pyrococcus

964 furiosus RAD50 (PDB: 1F2T and 1FTU). AlphaFold models used to facilitate structural predictions:

965 human SMC5 (AF-Q8IY18-F1). Source data and uncropped and unprocessed scans of the

966 immunoblotting experiments are provided with this paper.

967

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Author contributions

L.J.G, J.J.R, R.F.S, R.M.B, R.H, G.S.M, S.L.C, M.R.H, N.M, A-K.B and G.S.S designed and performed the cell biology experiments. F.U, X.L, T.K, and E.E.D generated, performed, and supervised analysis on the *slf2* and *smc5* knockdown and knockout zebrafish. L.J.G, A.G and S.S.J designed and generated the *SLF2* CRISPR knockout constructs and U-2 OS cell lines. A.W.O carried out structural modelling of patient-associated mutations in SMC5. T.N. and N.Matsumoto, designed and generated aid-tagged SLF2 degron cell lines. B.I, G.A.M-M, S.C, C.G.M, D.P, M.A.S, N.N, Z.Y, M.D, A.K, P.V, A-M.J, S.A.S, C.G-J, K.W.B, A.P.A.S, M.K, D.J, Y.U, Y.O, A.M, H.O, Z.A, J.A, C.T.R.M.S, A.M.R.T, A.P.J and C.Le C provided patient samples, performed next-generation sequencing, carried out bioinformatic analysis of next-generation sequencing data and performed other molecular genetic experiments. J.J.R,

1161   A.M.R.T, N.M, A-K.B, A.P.J, E.E.D. and G.S.S wrote the manuscript. G.S.S. planned and supervised

1162   the study.

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1164   **Competing Financial Interests**

1165   The authors declare no competing financial interests.

1166

1167   **Figure Legends**

1168

1169   **Figure 1: *SLF2* and *SMC5* variants cause severe microcephaly and short stature**

1170   **a** Table listing biallelic *SLF2* and *SMC5* variants in 11 individuals**.** ss, splice site created or destroyed

1171   by variant. '-' denotes that the allele variant was not present in the gnomAD database. Scores predicting

1172   the pathogenicity of the identified missense variants in *SLF2* and *SMC5* were generated using

1173   Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/). NA; Not applicable. **b** Length and head

1174   circumference (occipital frontal circumference; OFC) at birth and at the age of last exam as z-scores

1175   (s.d. from population mean for age and sex; SD). Dashed line at -3 SD indicates cut-off for normal

1176   population distribution. Orange values indicate SMC5 patients and blue values indicate SLF2 patients.

1177   **c** Schematic of full length WT SLF2 protein and SLF2 patient variants. APIM, atypical PCNA binding

1178   motif. SMC, SMC5/6 binding region. SLF1, SLF1 binding region. **d** Schematic of full length WT SMC5

1179   protein and SMC5 patient variants. CC, coiled-coil region.

1180

1181   **Figure 2: Impact of patient associated variants on the stability of SLF2 and SMC5 protein and**

1182   **the integrity of the SMC5/6 complex**

1183   **a** Representative immunoblot analysis of cell extracts from lymphoblastoid (LCL) cell lines derived from

1184   patients with variants in *SLF2*. WT-AH and WT-LQ (WT; wild type) indicate unrelated heathy individuals.

1185   **b** Representative immunoblot analysis of cell extracts from lymphoblastoid (LCL) cell lines derived from

1186   patients with variants in *SMC5*. WT-SW and WT-WCS indicate unrelated heathy individuals. **c & d**

1187   Whole cell extracts prepared from WT cell lines, SLF2 patient LCLs (c) or SMC5 patient LCLs (d) were

1188   subjected to immunoprecipitation with the indicated antibodies, and inputs and immunoprecipitates (IP)

1189   were analysed by immunoblotting (IB). **e** U-2 OS cells expressing Flag-SLF2 were transfected with WT

1190   or mutant GFP-SMC5. GFP-SMC5 was precipitated from cell extracts using GFP-Trap beads and co-

1191   precipitated proteins were detected using immunoblotting with the indicated antibodies. * represents a

1192   cross-reaction of the NSMCE2 antibody to GFP. **f** Whole cell extracts prepared from WT cell lines or

1193   SMC5 patient LCLs were subjected to immunoprecipitation with the indicated antibody, and inputs and

1194   immunoprecipitates were analysed by immunoblotting. Immunoblotting and immunoprecipitation

1195   experiments in panels a, b, c, d, and f are representative of two independent experiments with similar

1196   results. Panel e is representative of three independent experiments with similar results.

1197

1198 **Figure 3: Loss of *slf2* and *smc5* in zebrafish give rise to microcephaly and aberrant craniofacial**

1199 **patterning**

1200 **a** Top: Representative lateral bright field images acquired at 3 days post-fertilization (dpf); white dashed

1201 shape depicts head size measured. Bottom: Representative ventral images of GFP signal from the

1202 anterior region of *-1.4col1a1:egfp* transgenic reporter larvae at 3 dpf. The white dashed lines show the

1203 ceratohyal angle. **b** Quantification of lateral head size measurements. Larvae were injected with two

1204 independent sgRNAs targeting *slf2* with or without Cas9; n=3 independent experiments (left to right;

1205 56, 37, 37, 36, 36 larvae/batch). **c** Quantification of the ceratohyal angle. Larvae were injected with two

1206 independent *slf2* sgRNAs: n=3 independent experiments (left to right; 39, 42, 30, 20, 44 larvae/batch).

1207 **d** Top: Representative lateral bright field images at 3 dpf. Bottom: Representative ventral images of

1208 GFP signal in the anterior region of *-1.4col1a1:egfp smc5* sgRNA1 transgenic larvae at 3 dpf. **e**

1209 Quantification of lateral head size measurements in 3 dpf larvae (as shown in panel A); n=3 independent

1210 experiments (left to right; 50, 50, 52, 46, 53, 38 larvae/batch). The chart shows two independent

1211 experiments for sgRNA1 and sgRNA2 with a vertical line grouping independent controls with test

1212 conditions. **f** Quantification of the ceratohyal angle. Larvae were injected with two independent *smc5*

1213 sgRNAs: n=3 independent experiments (left to right; 34, 53, 37, 62, 28, 48 larvae/batch). The chart

1214 shows two independent experiments for sgRNA1 and sgRNA2 with a vertical line grouping independent

1215 controls with test conditions. **g** Left: Representative lateral bright field images of WT control and *slf2*^{-/-}

1216 mutants at 3 dpf. Right: Quantification of lateral head size measurements in 3 dpf WT control and *slf2*[-]

1217 [/-] mutant larvae (as shown in panel a); n=3 independent experiments (left to right; 10, 12, 12

1218 larvae/batch). In (a & b): (top left) white dashed shape depicts head size measured; (bottom left) white

1219 dashed lines show the ceratohyal angle measured. Abbreviations: MK, Meckel's cartilage; CH,

1220 ceratohyal cartilage (indicated with arrowheads, respectively); and CB, ceratobranchial arches

1221 (asterisks). Scale bars represent 300 μm, with equivalent sizing across panels. Error bars represent

1222 standard deviation of the mean. Statistical differences were determined with an unpaired Student's t-

1223 test (two sided).

1224

1225 **Figure 4: Loss of *slf2* and *smc5* induces apoptosis and altered cell cycle progression in**

1226 **zebrafish larvae**

1227 **a** Representative dorsal inverted fluorescent images showing TUNEL positive cells in control and *slf2*

1228 F0 mutants at 2 dpf (left two panels), and control and *smc5* F0 mutants at 3 dpf (right two panels). The

1229 blue dashed line indicates the region of interest (ROI) quantified. Embryos of the same developmental

1230 stage and similar magnification were evaluated for all *slf2* and *smc5* conditions. **b** Left: Quantification

1231 of TUNEL positive cells in the ROI of control and *slf2* F0 mutants at 2 dpf shown in panel a (left to right;

1232 27, 23, 19, 29, 30 embryos/condition were analysed from 3 independent experiments). Right:

1233 Quantification of TUNEL positive cells in control and *smc5* F0 mutants at 3 dpf in the ROI as shown in

1234 panel a (left to right; 37, 27, 22, 25, 23, 23 embryos/condition were analysed from 3 independent

1235 experiments). The chart shows two independent experiments for sgRNA1 and sgRNA2 with a vertical

1236 line grouping independent controls with test conditions. **c** Representative dorsal inverted fluorescent

1237 images showing phospho-histone H3 (pHH3) positive cells in control and *slf2* F0 mutants at 2 dpf (left

1238 two panels), and control and *slf2* F0 mutants at 3 dpf (right two panels). Embryos of the same

1239 developmental stage and similar magnification were evaluated for all *slf2* and *smc5* conditions. **d** Left:

1240 Quantification of pHH3 positive cells of control and *slf2* F0 mutants at 2 dpf in the ROI as shown in

1241 panel a (left to right; 21, 24, 22, 24, 26 embryos/condition were analysed from 3 independent

1242 experiments). Right: Quantification of pHH3 positive cells in the ROI in control and *smc5* F0 mutants at

1243 3 dpf as shown in panel a (left to right; 25, 23, 26 embryos/condition were analysed from 3 independent

1244 experiments). For all panels: Statistical differences were determined with an unpaired Student's t-test

1245 (two sided). Error bars represent standard deviation of the mean. Scale bars, 30 μm with equivalent

1246 sizing across panels.

1247

1248 **Figure 5: Patient-derived cell lines from individuals with bi-allelic *SLF2* or *SMC5* variants exhibit**

1249 **increased levels of spontaneous replication fork instability**

1250 **a** Top: Schematic representation for DNA fibre analysis in untreated cells. The indicated cell lines were

1251 pulse-labelled with CldU for 20 min, then pulse-labelled with IdU for 20 min. Bottom: DNA fibre analysis

1252 of SLF2 patient-derived LCLs or LCLs from a WT individual. The percentage of ongoing forks (left) or

1253 stalled forks (right) was quantified. n=4 independent experiments. A minimum of 1,500 fork structures

1254 were counted. **b** DNA fibre analysis of SMC5 patient-derived LCLs or WT LCLs. Quantification of the

1255 levels of ongoing forks (left) or stalled forks (right). n=4 independent experiments. A minimum of 750

1256 fork structures were counted. **c & d** Quantification of replication fork asymmetry of WT, SLF2 patient

(c) or SMC5 patient LCLs (d). n=4 independent experiments. A minimum of 75 fork structures were

1258 counted. Red lines denote median values. A Mann-Whitney rank sum test was performed for statistical

1259 analysis. Replication fork asymmetry represents the ratio of the left to right fork-track lengths of

1260 bidirectional replication forks. **e & f** DNA fibre analysis of SLF2 (e) and SMC5 (f) mutant fibroblast cell

1261 lines infected with lentiviruses encoding WT SLF2, WT SMC5, or an empty vector. The percentage of

1262 ongoing forks (left) or stalled forks (right) in untreated cells was quantified. A minimum of 350 fork

1263 structures in total were counted over 3 independent experiments. **g** DNA fibre analysis of U-2-OS SLF2

1264 CRISPR hypomorphic (HM) cells infected with lentiviruses encoding WT SLF2 or an empty vector. The

1265 percentage of stalled forks in untreated cells was quantified. A minimum of 1,000 fork structures in total

1266 were counted over 3 independent experiments. For panels a, b, e, f and g; a Student's t-test (two-sided,

1267 equal variance) was performed for statistical analysis and error bars denote SEM.

1268

1269 **Figure 6: SLF2 and SMC5 patient cells exhibit S-phase associated DNA damage**

1270 **a** Percentage of cells positive for EdU staining with >10 53BP1 foci in SLF2 and SMC5 mutant fibroblast

1271 cell lines infected with lentiviruses encoding WT SLF2, WT SMC5, or an empty vector. A minimum of

1272 900 EdU positive cells across 3 independent experiments were counted. **b** SLF2 and SMC5 patient

1273 fibroblast cell lines were pulsed with 10 µM EdU for 45 min, fixed, and mitotic DNA synthesis was

1274 visualised by mitotic EdU incorporation following labelling with click chemistry. The percentage of mitotic

1275 cells with EdU foci was quantified. A minimum of 300 mitotic cells were counted. n=3 independent

1276 experiments. **c** Immunofluorescent microscopy analysis to quantify the percentage of G1-phase cells

1277 (CENPF negative cells) with >3 53BP1 bodies in WT SLF2, WT SMC5, or an empty vector expressing

1278 SLF2 and SMC5 patient fibroblasts. n=3 independent experiments. A minimum of 750 G1-phase cells

1279 were counted. **d** Levels of micronuclei in cells from (c). n=3 independent experiments. A minimum of

1280 2,500 cells were counted. **e** Levels of micronuclei in U-2 OS SLF2 CRISPR HM cells infected with

1281 lentiviruses encoding WT SLF2 or an empty vector. n=3 independent experiments. A minimum of 1,700

1282 cells were counted. **f & g** Quantification of the average number of chromosomal aberrations per

1283 metaphase (which includes chromatid/chromosome gaps, breaks, fragments and chromosomes

1284 radials) in WT, SLF2 patient (f), or SMC5 patient LCLs (g). n=3 independent experiments. A minimum

1285 of 140 metaphases were counted. **h** Average number of chromosomal aberrations per metaphase

1286 (chromatid/chromosome gaps, breaks, fragments and chromosome radials) in SLF2 and SMC5 mutant

1287 fibroblast cell lines infected with lentiviruses encoding WT SLF2, WT SMC5, or an empty vector was

1288 quantified. n=3 independent experiments. A minimum of 90 metaphases were counted. **i** Average

1289 number of chromosomal aberrations (chromatid/chromosome gaps, breaks, fragments and

1290 chromosome radials) per metaphase in U-2 OS SLF2 CRISPR HM cell lines expressing either WT SLF2

1291 or an empty vector. n=3 independent experiments. A minimum of 100 metaphases were counted. In all

1292 cases, a Student's t-test (two-sided, equal variance) was performed for statistical analysis and error

1293 bars denote SEM.

1294

1295 **Figure 7: SLF2 and SMC5 patient cells exhibit mosaic variegated hyperploidy, mitotic**

1296 **abnormalities and sister chromatid cohesion defects**

1297 **a** Quantification of the numbers of chromosomes per metaphase in peripheral blood lymphocytes from

1298 SLF2 or SMC5 patients, or an unrelated WT individual. 200 metaphases were counted in total from 2

1299 independent blood samples. **b** Average number of mitotic cells with mis-segregated lagging

1300 chromosomes in SLF2 and SMC5 mutant fibroblast cell lines infected with lentiviruses encoding WT

1301 SLF2, WT SMC5, or an empty vector. n=3 independent experiments for SLF2-P1, SMC5-P7 and SMC5-

1302 P8, and n=4 independent experiments for SLF2-P2. A minimum of 250 mitotic cells were counted. **c**

1303 Representative images of mitotic cells from (b) with lagging chromosomes (scale bar: 10 µM). **d**

1304 Average number of mitotic cells with mis-segregated lagging chromosomes in U-2 OS SLF2 CRISPR

1305 HM cells infected with lentiviruses encoding WT SLF2 or an empty vector. n=3 independent

1306 experiments. A minimum of 190 mitotic cells were counted. **e** Left: percentage of metaphases with rail

1307 road chromosomes in peripheral blood lymphocytes from SLF2 or SMC5 patients, or an unrelated WT

1308 individual. A minimum of 380 metaphases were counted in total from 2 independent blood samples.

1309 Right: Representative images of metaphases (scale bar: 10 µM). **f** Percentage of metaphases with

1310 premature chromatid separation following 4 h treatment with 25 µM MG132 in SLF2 and SMC5 patient

1311 LCLs. n=4 independent experiments. 200 total metaphases were counted. **g** Percentage of S/G2 cells

1312 (CENPF positive cells) with >2 centrosomes with or without 24 h exposure to 250 nM APH. n=3

1313 independent experiments. A minimum of 900 CENPF positive cells were counted. **h** Percentage of

1314 mitotic cells in SLF2 and SMC5 mutant LCLs with multi-polar spindles in untreated cells and cells

1315 exposed to 250 nM APH for 24 h. A minimum of 300 mitotic cells were counted over 3 independent

1316 experiments. **I** The percentage of G1-phase cells (CENPF negative cells) with >5 53BP1 bodies in SLF2

1317 and SMC5 mutant fibroblast cell lines, with or without 24 h exposure to 500nM APH. n=4 independent

1318 experiments. A minimum of 390 G1-phase cells were counted. In all cases, a Student's t-test (two-

1319 sided, equal variance) statistical test was performed and error bars denote SEM.

1320

1321 **Figure 8: Variants in the RAD18-SLF1/2-SMC5/6 complex compromise the ability of cells to**

1322 **replicate in the presence of stabilised G4 quadruplex structures.**

1323 **a** Left: Average number of segmented chromosomes per metaphase in peripheral blood lymphocytes

1324 (PBLs) from SLF2 or SMC5 patients, or an unrelated WT individual. 250 total metaphases were counted

1325 from 2 independent blood samples. Middle: Representative images of 'type 1' and 'type 2' segmented

1326 chromosomes. Right: Representative image of a metaphase exhibiting segmented chromosomes from

1327 SLF2-P3 PBLs (scale bar: 10 µM). **b** Representative image of FISH with a centromere-specific probe

1328 showing di-centric chromosomes in a metaphase prepared from SLF2-P3 PBLs (scale bar: 10 µM). **c**

1329 Average number of sister chromatid exchanges in metaphase spreads from SLF2 and SMC5 patient-

1330 derived LCLs. n=3 independent experiments. A minimum of 100 metaphases were counted. **d**

1331 Quantification of the IdU:CldU track length ratio in untreated and CX451-treated SLF2 and SMC5

1332 patient fibroblast cells. Cell lines were pulse-labelled first with CldU for 30 min, followed by IdU, with or

1333 without 250 nM CX5461, for 30 min. n=3 independent experiments. A minimum of 250 ongoing fork

1334 structures were counted. **e** Average number of chromosomal aberrations (chromatid/chromosome

1335 gaps, breaks, fragments and chromosome radials) per metaphase in SLF2 and SMC5 patient-derived

1336 LCLs with and without 24 h exposure to 250 nM CX5461. n=5 independent experiments. A minimum of

1337 350 metaphases were counted. Student's t-test (two-sided, equal variance) was performed. Error bars

1338 denote SEM. **f** LCL proliferation assay. WT and SLF2 and SMC5 patient-derived LCLs were cultured in

1339 increasing concentrations of CX5461 for the time untreated cells took to undergo three population

1340 doublings. Cell viability following CX5461 treatment was calculated as a percentage of the number of

1341 untreated cells. n=4 independent experiments. Error bars denote SEM. A two-way ANOVA statistical

1342 test was performed. **g** Quantification of IdU:CldU track length ratio in untreated, pyridostatin-, etoposide-

1343 and BMH21-treated SLF2 and SMC5 mutant fibroblast cells. Cell lines were pulse-labelled first with

1344 CldU for 30 min, followed by IdU with or without 1µM pyridostatin, 50nM etoposide or 1 µM BMH21, for

1345 30 min. n=3 independent experiments. A minimum of 150 ongoing forks were counted. For panels c, d

1346 and g, red lines denote median values, and a Mann-Whitney rank sum statistical test was performed.

Figure 1

a

Individual	Ancestry	Gene	Mutation 1	Allele Frequency	Polyphen-2 score	Mutation 2	Allele Frequency	Polyphen-2 score
P1	UK	*SLF2*	c.1006dup; p.(Arg336LysfsTer27)	-	NA	c.1006dup; p.(Arg336LysfsTer27)	-	NA
P2	France	*SLF2*	c.2444C>G; p.(Ser815Ter)	-	NA	c.3486G>C (ss); p.(Gln1162His)	-	0.999
P3	Netherlands	*SLF2*	c.3330G>A (ss) p.(Arg1110ArgΔexon17)	-	NA	c.3330G>A (ss); p.(Arg1110ArgΔexon17)	-	NA
P4-1	Japan	*SLF2*	c.2582A>T; p.(Asn861Ile)	3.98e-6	0.996	c.2719dup; p.(Ser907PhefsTer5)	-	NA
P4-2	Japan	*SLF2*	c.2582A>T; p.(Asn861Ile)	3.98e-6	0.966	c.2719dup; p.(Ser907PhefsTer5)	-	NA
P5	Saudi Arabia	*SLF2*	c.2347_2348del; p.(Asp783SerfsTer53)	-	NA	c.2347_2348del; p.(Asp783SerfsTer53)	-	NA
P6	German	*SLF2*	c.568C>T; p.(Arg190Ter)	3.19e-5	NA	c.568C>T; p.(Arg190Ter)	3.19e-5	NA
P7	Spain	*SMC5*	c.1110_1112del; p.(Arg372del)	-	NA	c.1273C>T; p.(Arg425Ter)	3.99e-6	NA
P8	US	*SMC5*	c.2970C>G; p.(His990Asp)	-	1.000	c.2970C>G; p.(His990Asp)	-	1.000
P9-1	US	*SMC5*	c.2970C>G; p.(His990Asp)	-	1.000	c.2970C>G; p.(His990Asp)	-	1.000
P9-2	US	*SMC5*	c.2970C>G; p.(His990Asp)	-	1.000	c.2970C>G; p.(His990Asp)	-	1.000

Figure 2

Figure 3

a

Control   *slf2* sgRNA1+Cas9

MK   CH   CB
* * *

b

Head area (μm²)

P = 2.6E-08
P = 0.31
P = 0.00024
P = 6.22E-06
P = 3.48E-05

Control   sgRNA1 alone   sgRNA1+Cas9   sgRNA2 alone   sgRNA2+Cas9

c

Ceratohyal angle (degrees)

P = 4.34E-06
P = 0.62
P = 0.0002
P = 0.0002
P = 8.54E-05
P = 0.388

Control   sgRNA1 alone   sgRNA1+Cas9   sgRNA2 alone   sgRNA2+Cas9

d

Control   *smc5* sgRNA1+Cas9

e

Head area (μm²)

P = 6.33E-16
P = 1.45E-11
P = 0.165
P = 5.05E-28
P = 2.19E-09
P = 0.747

Control   sgRNA1 alone   sgRNA1+Cas9   Control   sgRNA2 alone   sgRNA2+Cas9

f

Ceratohyal angle (degrees)

P = 1.21E-05
P = 2.91E-09
P = 0.602
P = 5.86E-07
P = 2.39E-06
P = 0.025

Control   sgRNA1 alone   sgRNA1+Cas9   Control   sgRNA2 alone   sgRNA2+Cas9

g

WT control

slf2 $^{-/-}$

Head area (μm²)

P = 5.6E-06
P = 0. 502

WT Control   *slf2* +/-   *slf2* -/-

Figure 4

a

b

c

d

Figure 5

Figure 6

Figure 7

Figure 8

a Legend:
- ■ Type 2 chromosome
- ■ Type 1 chromosome with 1 break
- ■ Type 1 chromosome with 2 breaks

Y-axis: Average number of segmented chromosomes per metaphase (0 to 0.6)
X-axis: WT-GS, SLF2-P1, SLF2-P2, SLF2-P3, SMC5-P7

Type 1 'segmented chromosome' with 1 break

Type 2 'dicentric chromosome'

SLF2-P3

▶ segmented chromosomes

b Type 2 'dicentric chromosome'

c
Left plot — Y-axis: Number of SCEs per chromosome (0 to 0.4)
X-axis: WT-AH, SLF2-P1, SLF2-P2, ATR-Seckel
P < 0.001, P < 0.001, P < 0.001

Right plot — Y-axis: Number of SCEs per chromosome (0 to 0.4)
X-axis: WT-AH, SMC5-P7, SMC5-P8, SMC5-P9-1
P < 0.001, P < 0.001, P < 0.001

d
Untreated — Y-axis: Ratio of IdU/CldU tract length (0 to 3)
P = 0.074, P = 0.265
X-axis: + Vector, + WT SLF2 (SLF2-P1); + Vector, + WT SMC5 (SMC5-P8)

CX5461 — Y-axis: Ratio of IdU/CldU tract length (0 to 3)
P < 0.001, P < 0.001
X-axis: + Vector, + WT SLF2 (SLF2-P1); + Vector, + WT SMC5 (SMC5-P8)

e
Legend: ■ WT-GS ■ SLF2-P1 ■ SMC5-P8
Y-axis: Average number of chromosomal aberrations per metaphase (0 to 0.7)
X-axis: Untreated, CX5461
P = 2.35E-05, P = 9.17E-05, P = 2.37E-05, P = 0.00022

f
Y-axis: Viability (% of untreated) (10 to 100)
X-axis: CX5461 (nM) (0 to 300)
Legend: WT-GS, SLF2-P1, SMC5-P8
P = 0.0017, P = 0.00028

g
Y-axis: Ratio of IdU/CldU tract length (0 to 3.5)
Untreated: P = 0.851, P = 0.541
Pyridostatin: P < 0.001, P < 0.001
Etoposide: P = 0.925, P = 0.242
BMH21: P = 0.118, P = 0.442
X-axis groups: + Vector, + WT SLF2 (SLF2-P1); + Vector, + WT SMC5 (SMC5-P8) for each condition

Supplementary Figure 1

a

SLF2

p.(Ans861Ile)		
Human	SLSDVAAVFFNMGIDFRSLFPLEN	908
Chimpanzee	SLSDVAAVFFNMGIDFRSLFPLEN	908
Dog	SLSDIAAVFFNMGIDFRSLFPLEN	905
Mouse	SLSDIAAVFFNMGVGFGSLFPLET	898
Chicken	SLADVTTVLVNMGIRLRSLFPLQH	924
Zebrafish	SIRDITQVFLNMGASFISLFPLDV	708
p.(Gln1162His)		
Human	GKWQEIIQNCRPTQGQLHDFWVPD	1173
Chimpanzee	GKWQEIIQNCRPTQGQLHDFWVPD	1173
Dog	GKWQEIIQNCRPTQGQLHDFWVPD	1170
Mouse	GKWQEIIQNCRPTQGQLHDFWVPD	1163
Chicken	GRWQDLIQNSRLTQGKLHDFWEPD	1189
Zebrafish	TKWQVLLTRTRPQEGMLYDYWKPP	999

b

SMC5

p.(His990Asp)		
Human	SSMQCAGEVDLHTENEEDYDKYGIRIRVKFRSSTQLHELTPHHQSGGERSVSTMLYLMAL	1007
Chimpanzee	SSMQCAGEVDLHTENEEDYDKYGIRIRVKFRSSTQLHELTPHHQSGGERSVSTMLYLMAL	1007
Dog	SSMQCAGEVDLHTENEEDYDKYGIRIRVKFRSSTQLHELTPHHQSGGERSVSTMLYLMAL	1011
Mouse	SSMQCAGEVDLHTENEEDYDKYGIRIRVKFRSSTQLHELTPHHQSGGERSVSTMLYLMAL	1007
Chicken	SSMESVGEVDLHVENEEEYDKYGIRIRVKFHNFTDLHELTPYHQSGGEKSVSTVLYLMAL	971
Zebrafish	QSMQCAGEVDLHSENEEEYDKYGIRIQVQFRRNTRMHELTPHHQSGGERSVTTMLYLMSL	982
Drosophila	ESIEYVGEVVLSKTDKYDFDSYGIQIMVQFRRGLQLQPLDKFIQSGGERAVSIAIYSLSL	955
S.Cerevisiae	NNVGSAGAVRLEKP--KDYAEWKIEIMVKFRDNAPLKKLDSHTQSGGERAVSTVLYMIAL	1002
S.Pombe	SGMGYACEVRLGKS--DDYDKWYIDILVQFREEEGLQKLTGQRQSGGERSVSTIMYLLSL	982
p.(Arg372del)		
Human	IERKD--KHIEELQQALIVKQNEEL-D--RQRRI-GNTRKMIEDLQNELKTTEN------	392
Chimpanzee	IERKD--KHIEELQQALIVKQNEEL-D--RQRRI-GNTRKMIEDLQNELKTTEN------	392
Dog	IERKD--KQIEELQQALTVKQNEEH-D--RQRRI-SNTRKMIEDLQNELKTTEN------	396
Mouse	IERKD--RQIEELQQALTVKQNEEL-D--RQKRI-SNTRKMIEDLQSELKTAEN------	392
Chicken	LEMKD--KQISEINQALRMKKDEEV-D--RKKKI-LSAYKMIDEWNNELNTVTD------	371
Zebrafish	LELKN--KEVDDIKQDMSLKQTEEA-D--RQKRI-GHTQLMIRDLQKELQNMGT------	381
Drosophila	-AAID--GKMDSLKQGIYQKK----------------YELEQNIKKSRRTATE------	336
S.Cerevisiae	-----IFEKLNTIRDEVIKKKNQNEYYRGRTKKLQATIISTKEDFLRSQEILAQT--HLP	393
S.Pombe	LRARASFSNFMENEKKLYEKVN-------TNRTLLRNANLTLNEAQQSVKSLTERQGPRP	362

Supplementary Figure 2

a

Full length SLF2 →
p.(Arg1110ArgΔexon17) →

b

WT SLF2

Exon 16 | Exon 17
ATTTAGAACTTGAAAAGCAGGCATATTACCTGACCTACAT

p.(Arg1110ArgΔexon17) SLF2

Exon 16 | Exon 18
ATTTAGAACTTGAAAAGCAGAAACACTTTGTGCTACTCTG

c

SLF2 NM_0018121

Supplementary Figure 3

a

SLF2 (FAM178A)

Chromosome 10: 100,912,569-100,965,136, hg19, strand +

ENST00000238961.8 (NM_018121)

ENST00000370269.3 (NM_001136123)

b

SLF2
NM_0018121

c

Human pooled cDNA
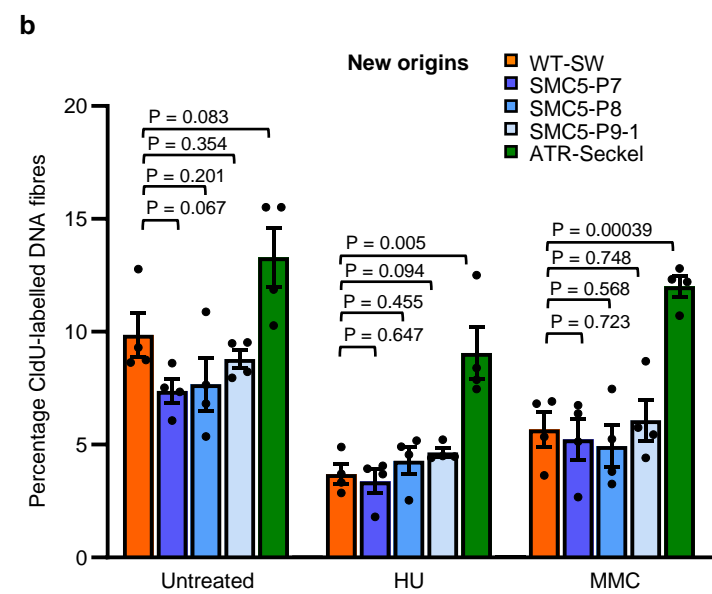
d

Human adult tissues

e

f

Supplementary Figure 4

Supplementary Figure 5

HINGE

Nse2; disordered
C-terminal tail

Arg365

Thr366

predicted
break in helix

Lys368

Ile744

*Sc.*Nse2
(3HTK)

*Sc.*Smc5
(3HTK + AlphaFold)

descending

ascending

HEAD

Arg369

Gln370

Leu728

Arg372

Arg371

*Hs.*Smc5
(AlphaFold)

a

Pf.Rad50 (1F2T)

Pf.Rad50 + ATP (1FTU)

*Hs.*SMC5 (AlphaFold)

b

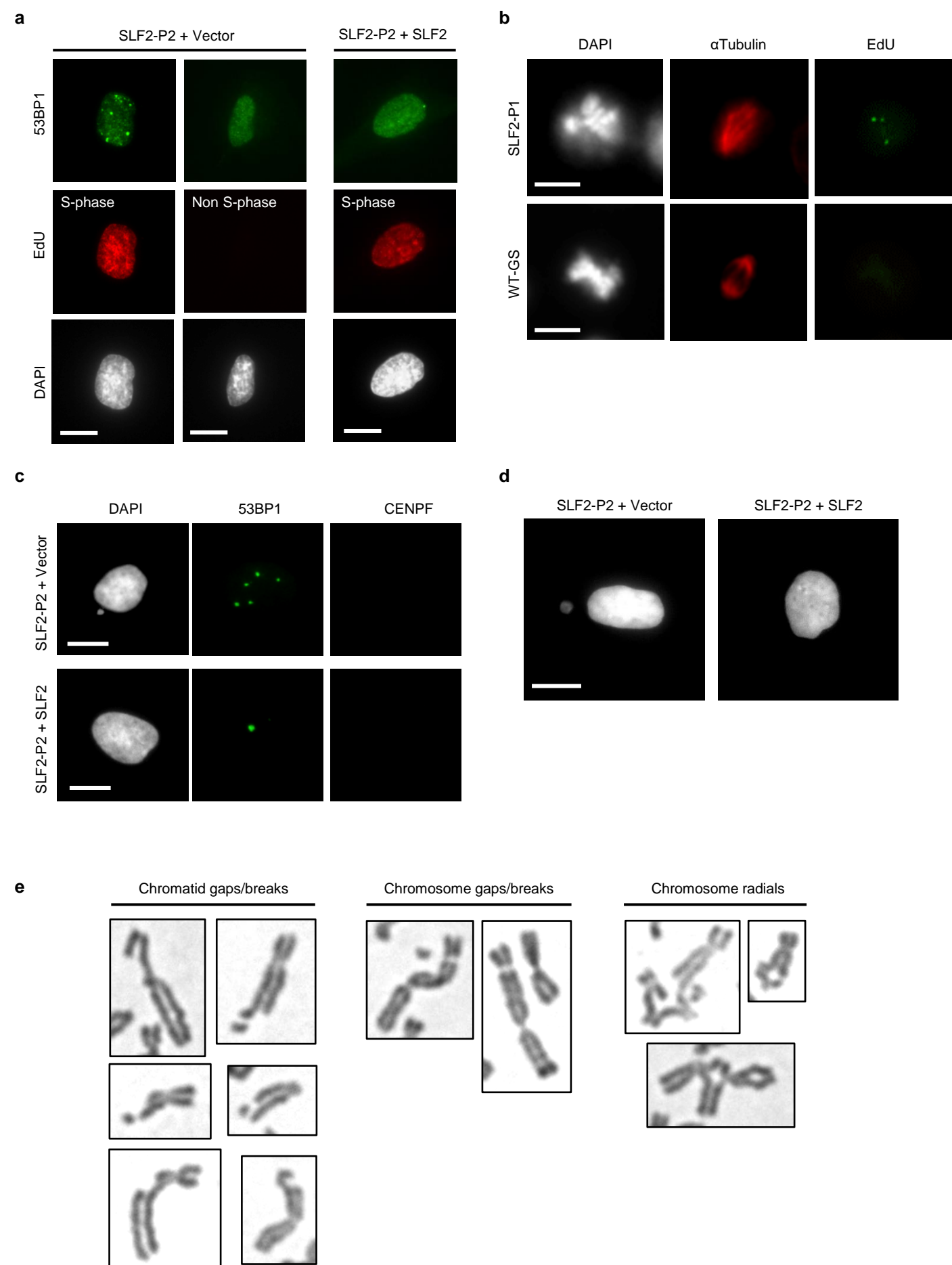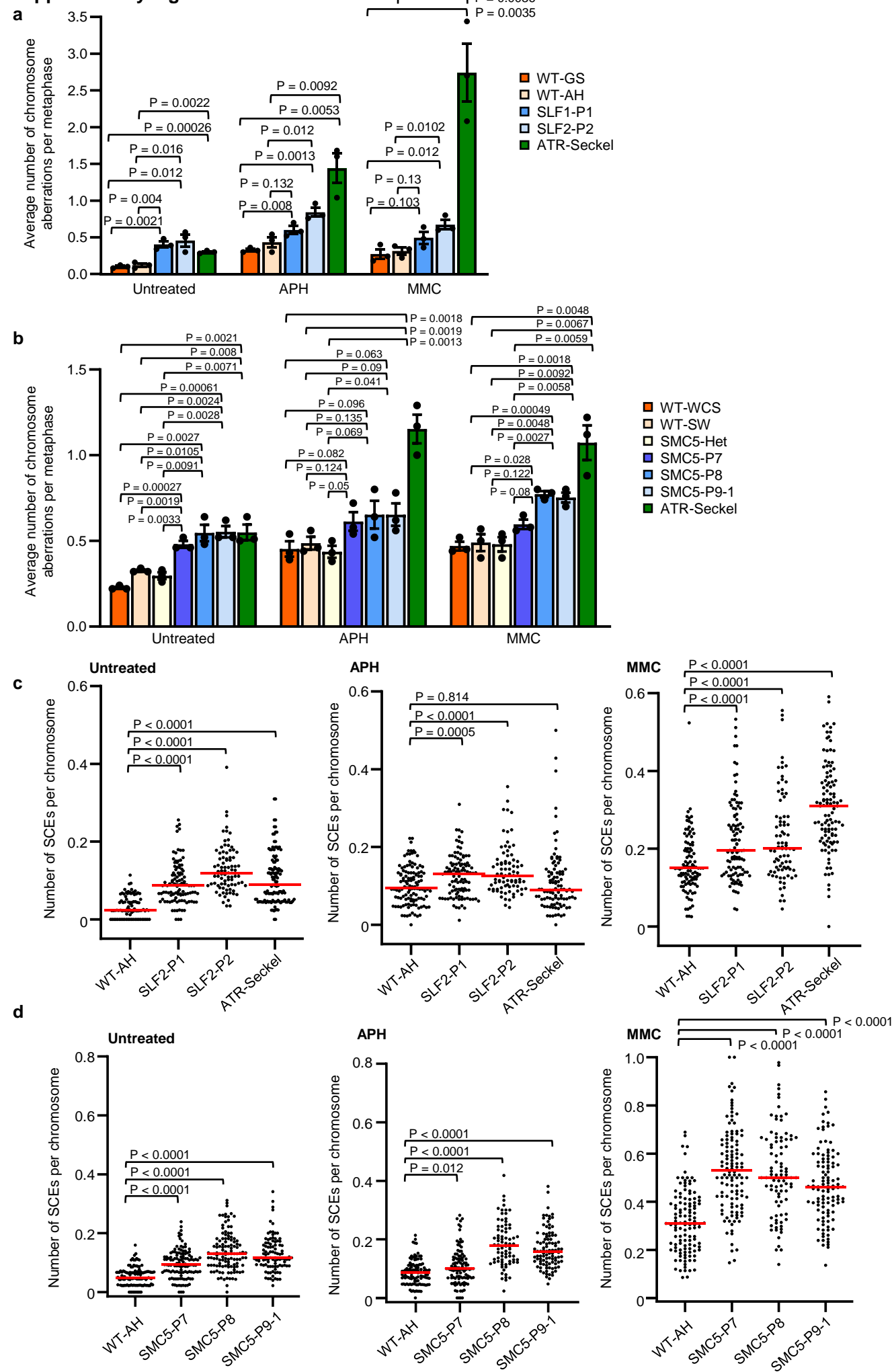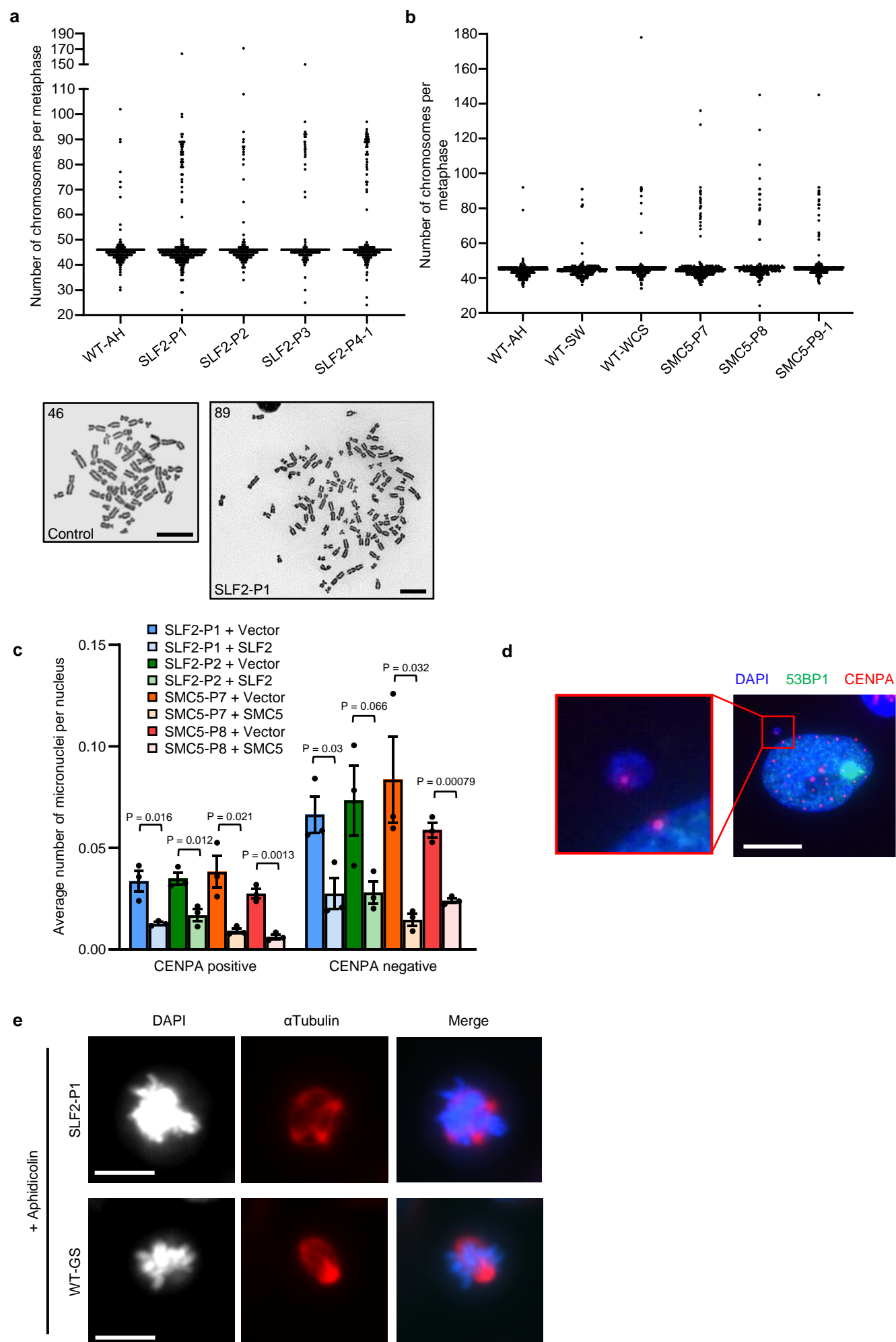| Analysis Program*	ΔΔG (kcal.mol^{-1})	Predicted Effect	
DynaMut	0.587	Stabilising	
mCSM	0.342	Stabilising	
DUET	0.368	Stabilising	
SDM	-0.200	Destabilising	
EnCOM	0.164	Destabilising	
Analysis Program	ΔΔSVib (kcal.mol^{-1}.K^{-1})	Predicted Effect	
ENCoM	-2.05	Decrease of molecule flexibility	
			* all values obtained from DynaMut webserver

Supplementary Figure 7
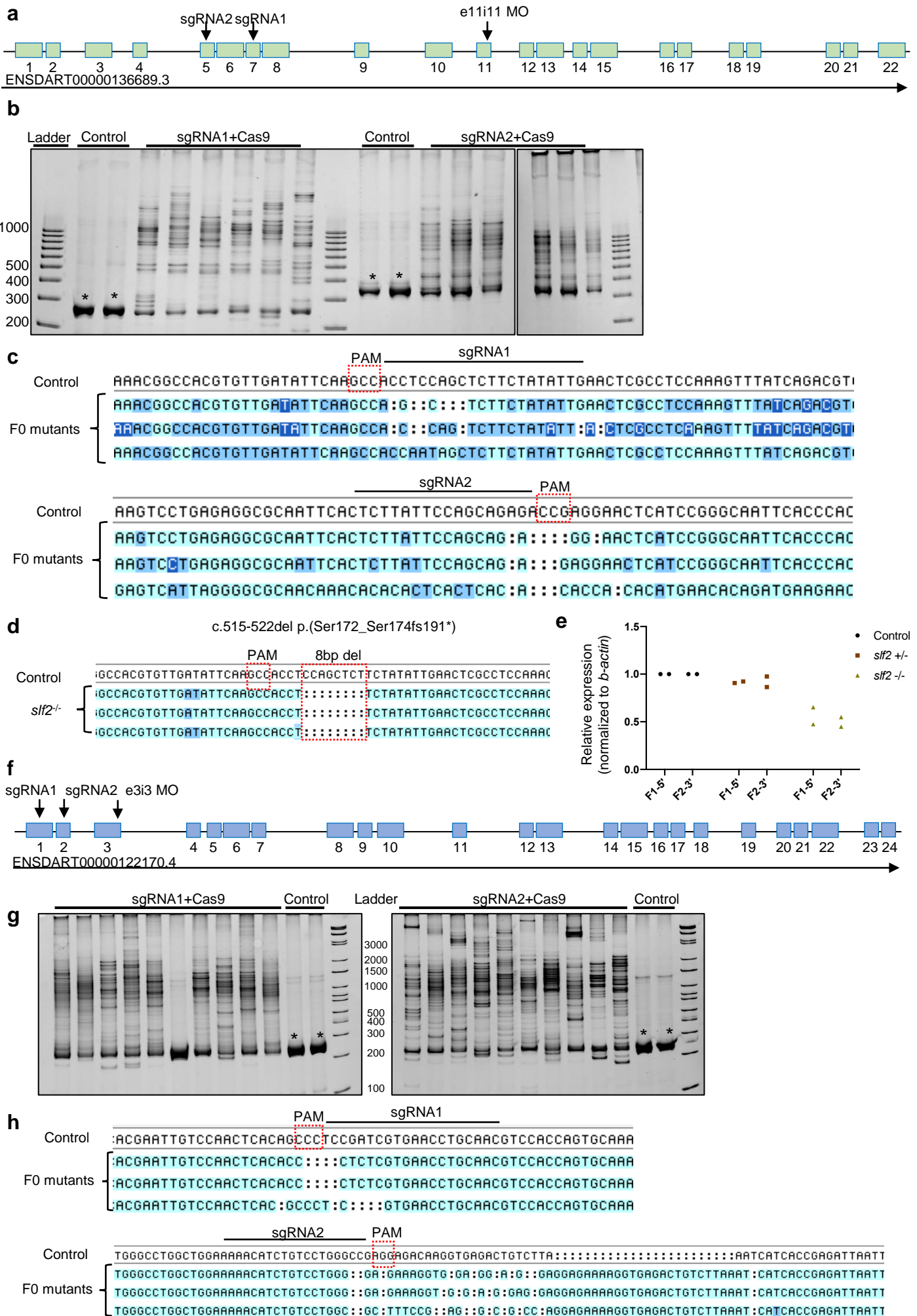
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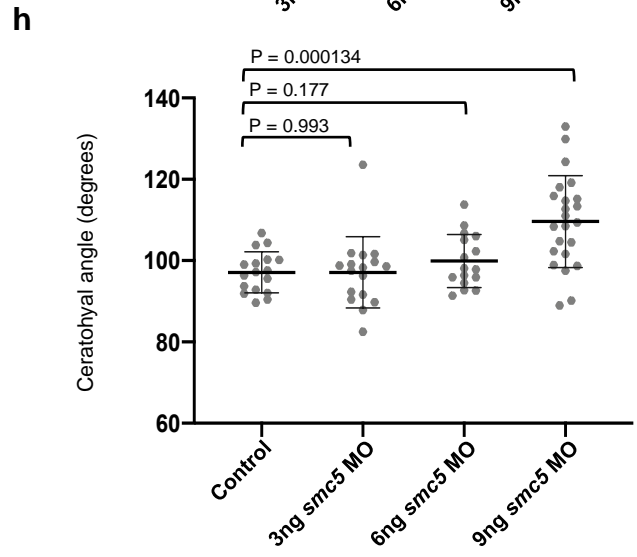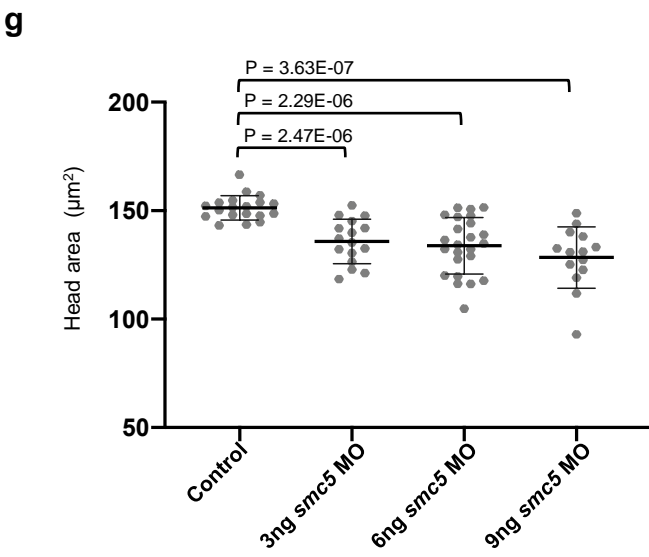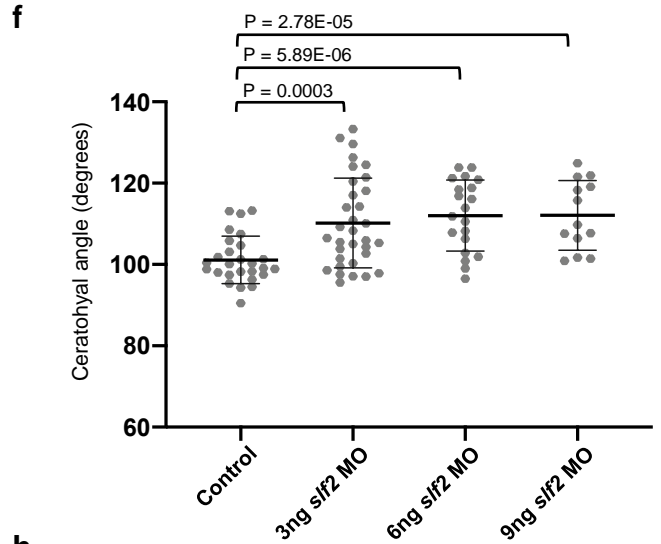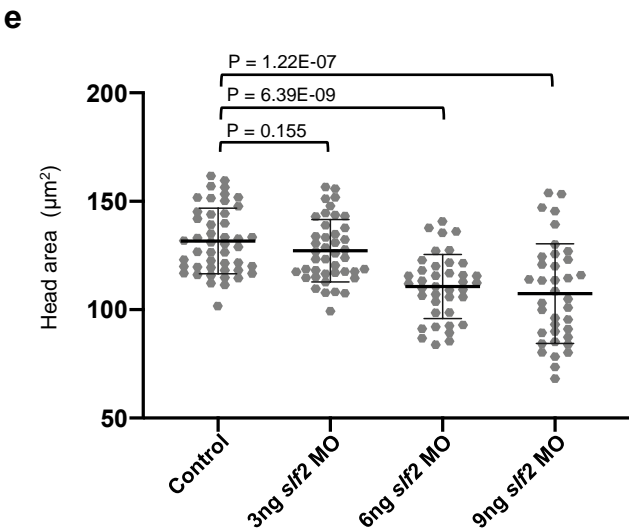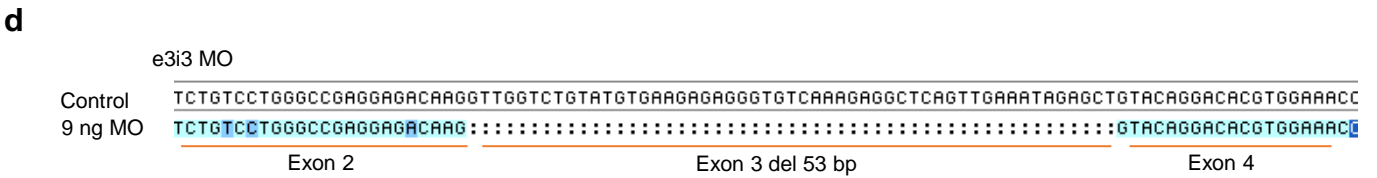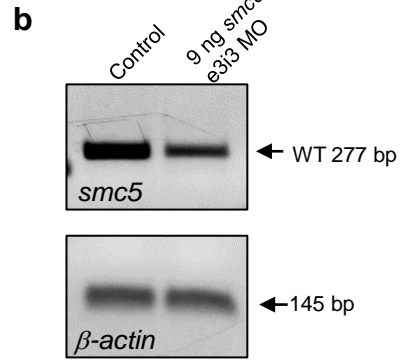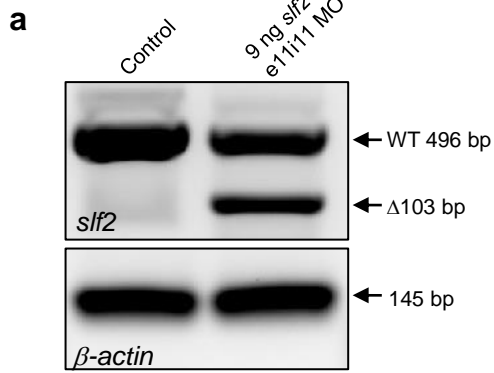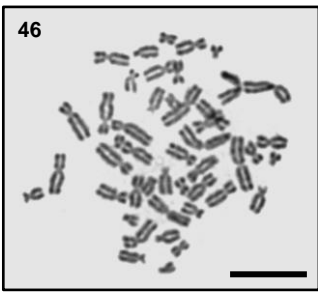
Supplementary Figure 9

Supplementary Figure 10

a

b

c

d

e

f

g

h
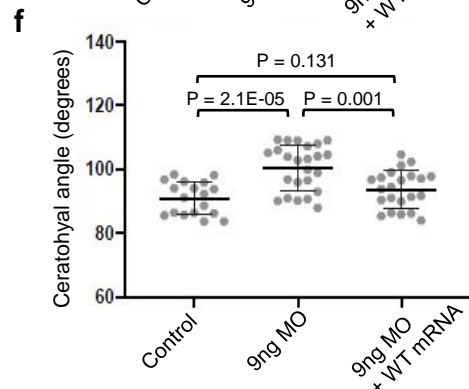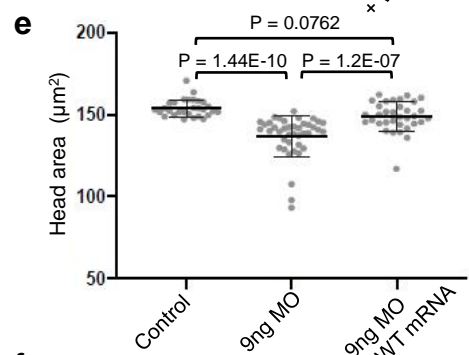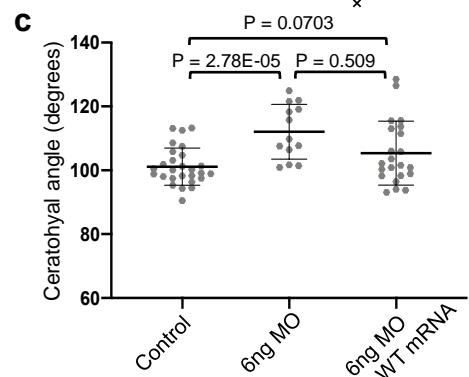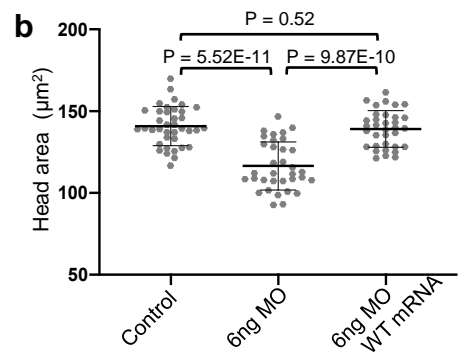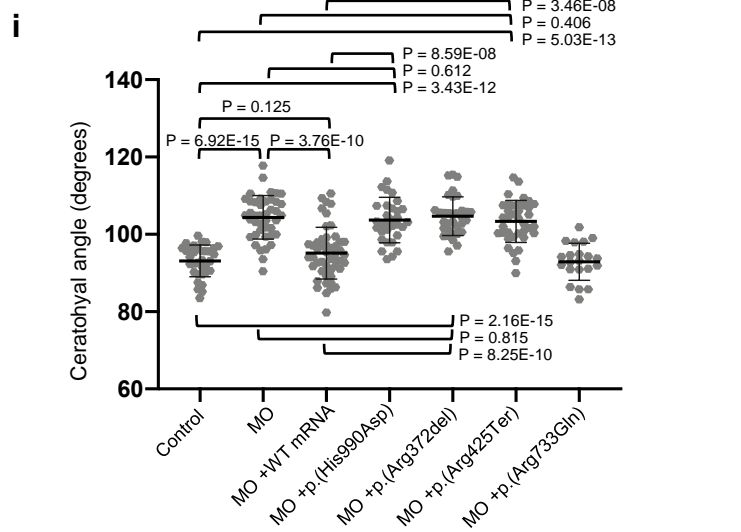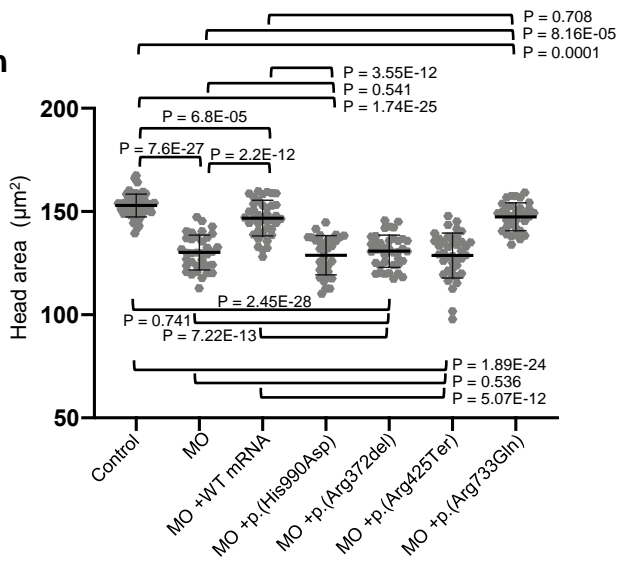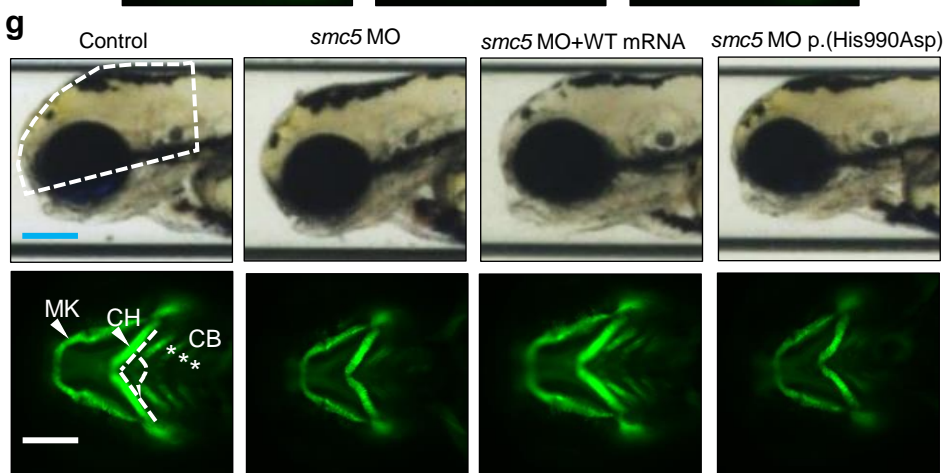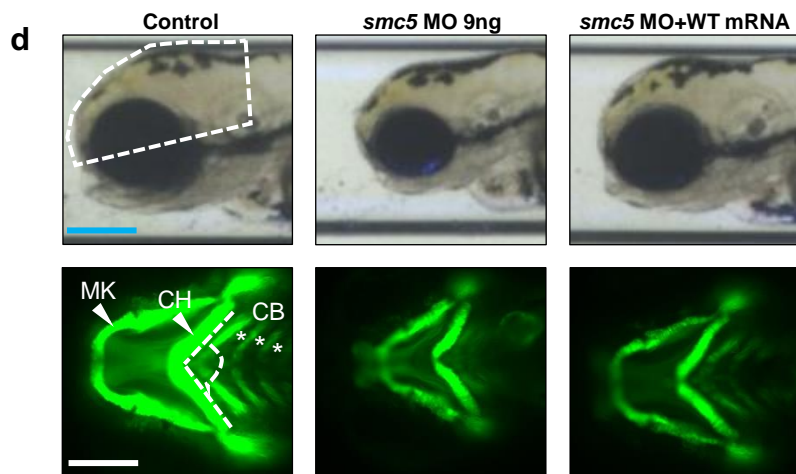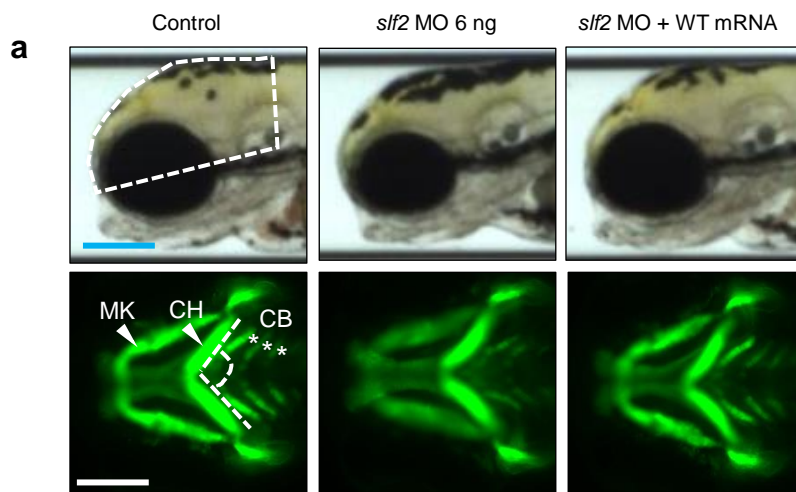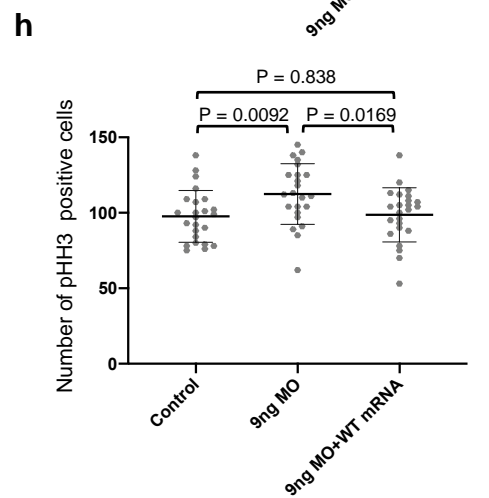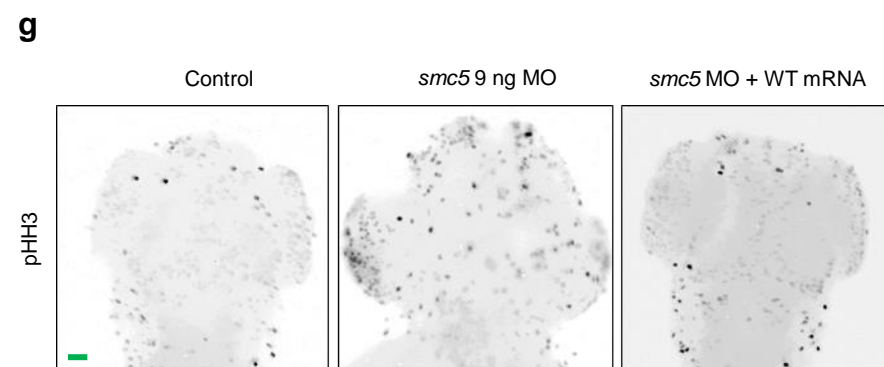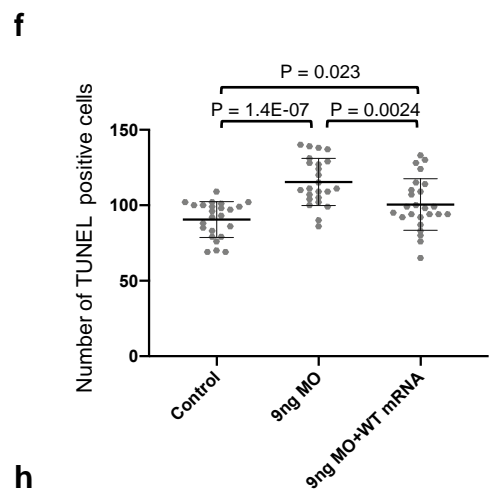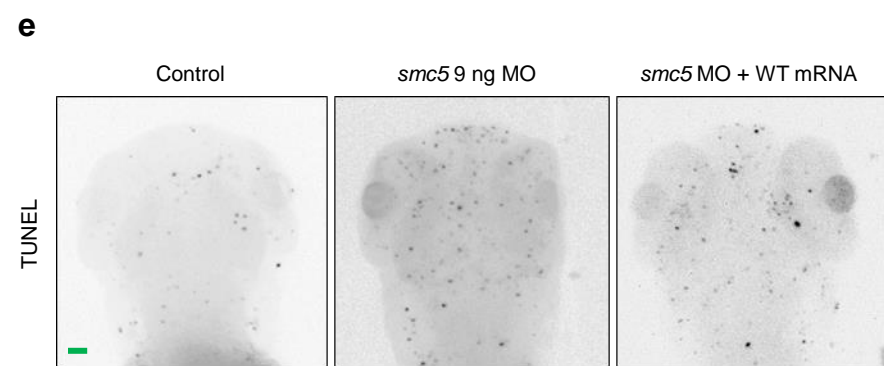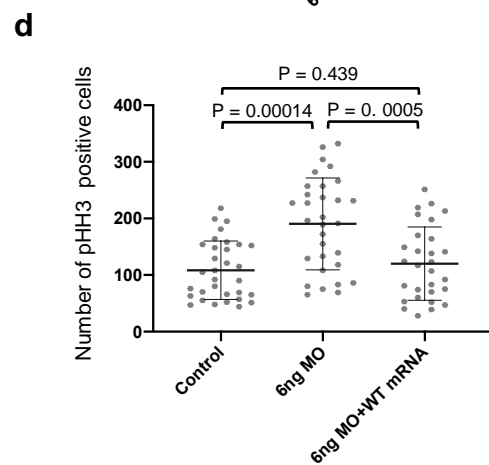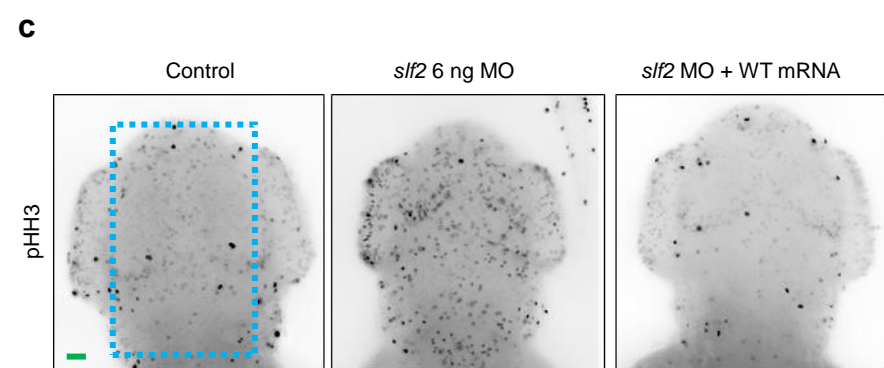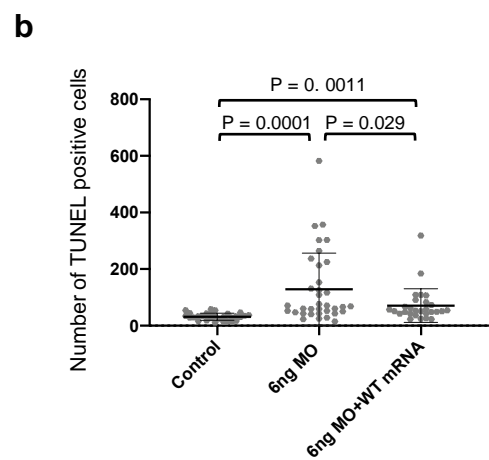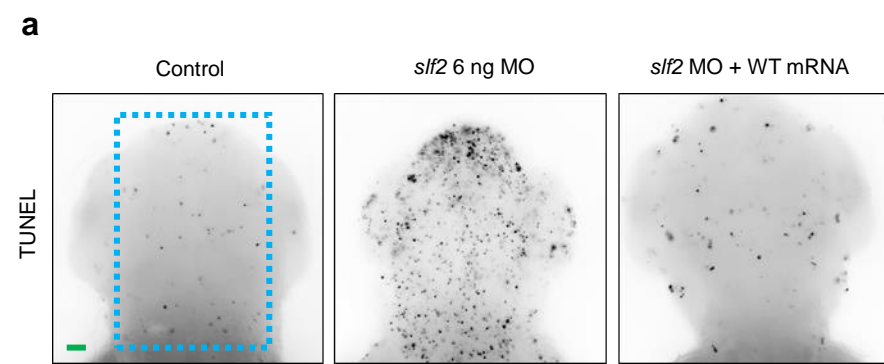
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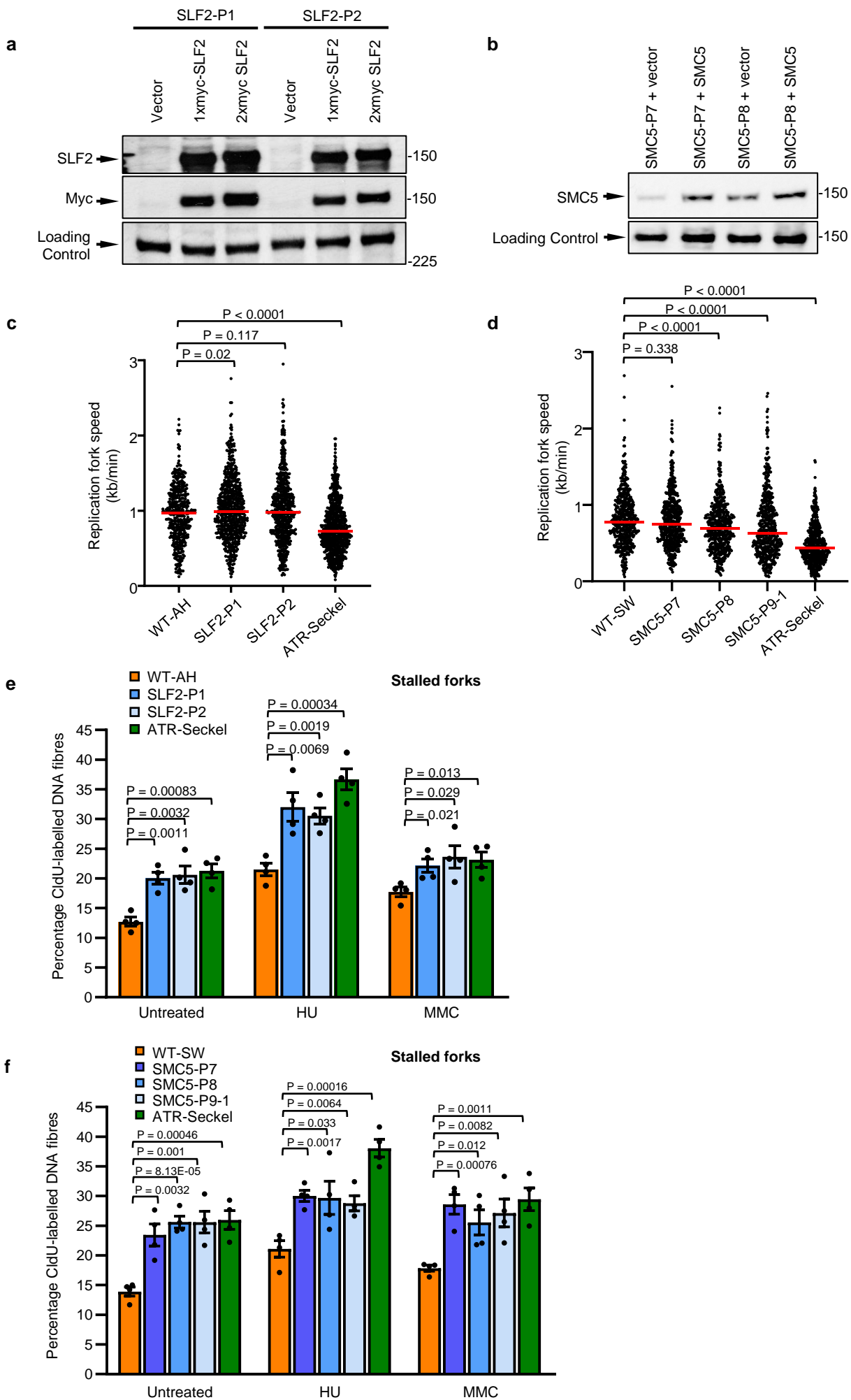
Supplementary Figure 12

a
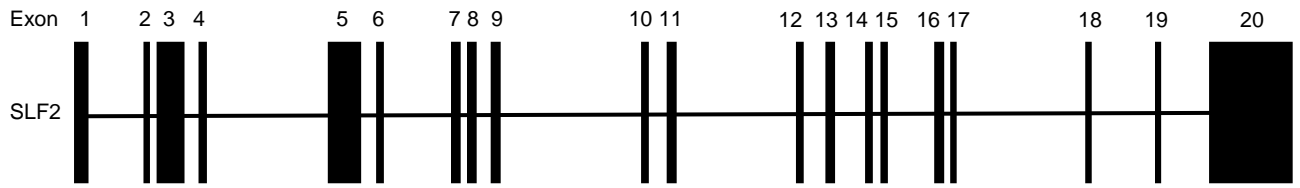

```
                                                    sgRNA 2
GAAGATATATCCAAGGAACCGAGTGATGAAACTGATGGCTCTTCTGCAGGCTTGGCACCTTCAAATTCTGGCAATTCTGG
CTTCTATATAGGTTCCTTGGCTCACTACTTTGACTACCGAGAAGACGTCCGAACCGTGGAAGTTTAAGACCGTTAAGACC
                      sgRNA 1
```

b

Clone #	Mutation
Cl. 1	c. 1192_1210dupGATGAAACTGATGGCTCTT, p.(Ser403Ter)
	c.1184_1232dupAACCGAGTGATGAAACTGATGGCTCTTCTGCAGGCTTGGCACCTTCAAA, p.(Asn411Lysfs3Ter)
	c.1185_1232dupACCGAGTGATGAAACTGATGGCTCTTCTGCAGGCTTGGCACCTTCAAA, p.(Ser410_Asn411insKPSDETDGSSAGLAPS)
Cl. 2	c.1207_1223delTCTTCTGCAGGCTTGGC, p.(Ser403Thrfs14Ter)
	c.1188_1208delGAGTGATGAAACTGATGGCTC, p.(Asp398_Ser404del)

c

d
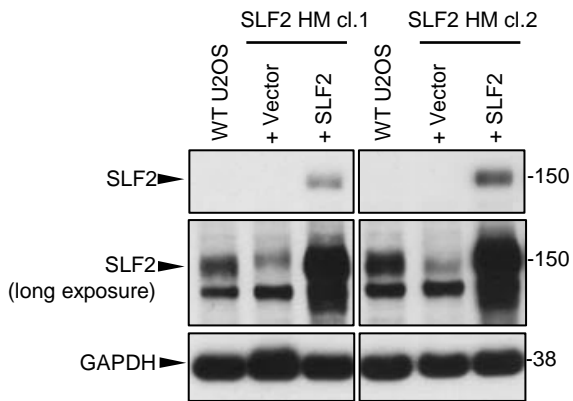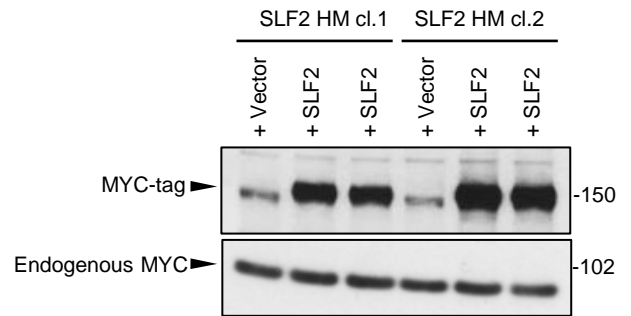
Supplementary Figure 13

Supplementary Figure 14

a

SLF2-P2 + Vector

SLF2-P2 + SLF2

53BP1

EdU

S-phase   Non S-phase   S-phase

DAPI

b

DAPI   αTubulin   EdU

SLF2-P1

WT-GS

c

DAPI   53BP1   CENPF

SLF2-P2 + Vector

SLF2-P2 + SLF2

d

SLF2-P2 + Vector   SLF2-P2 + SLF2

e

Chromatid gaps/breaks   Chromosome gaps/breaks   Chromosome radials
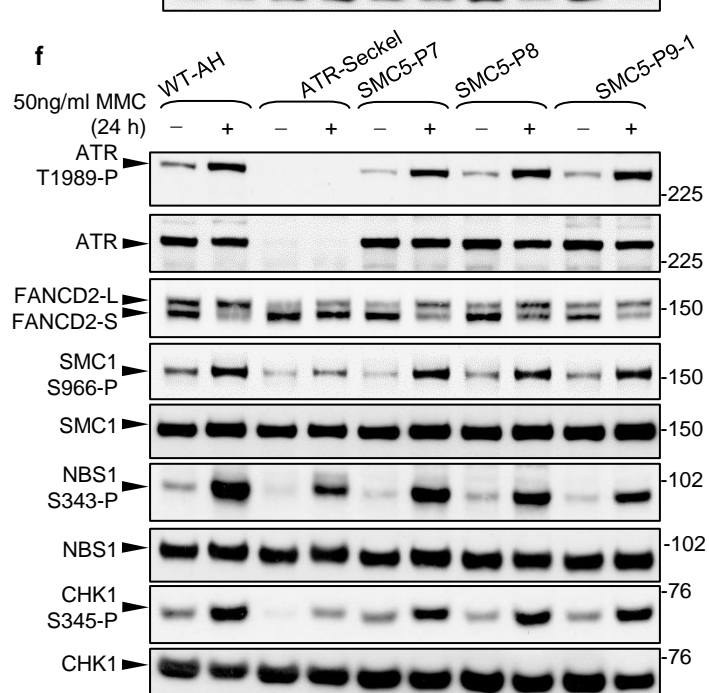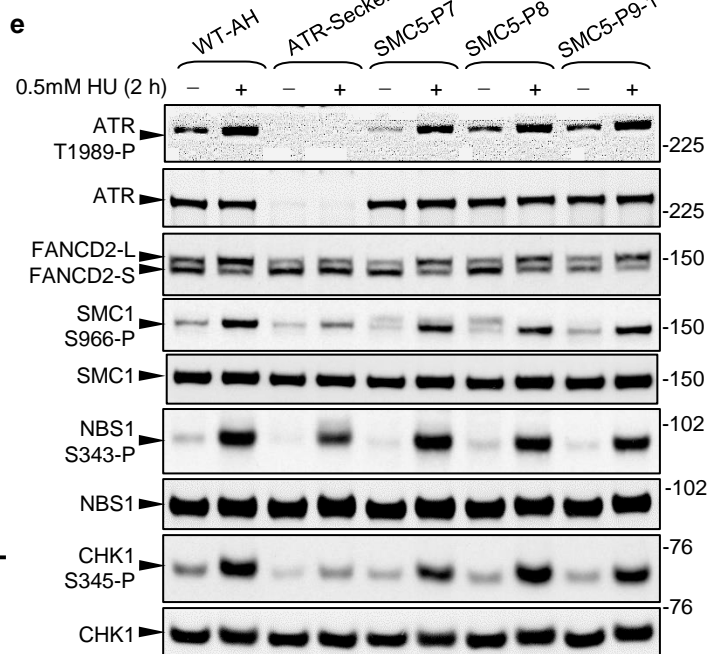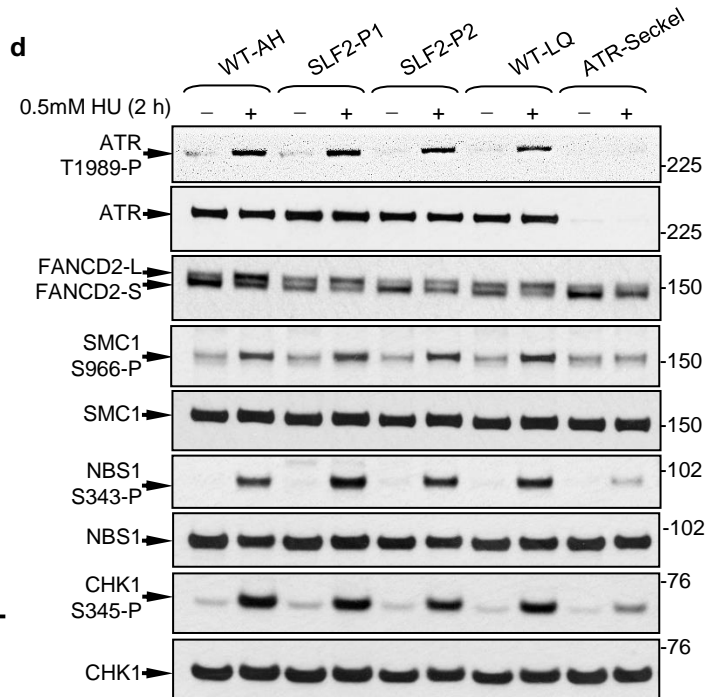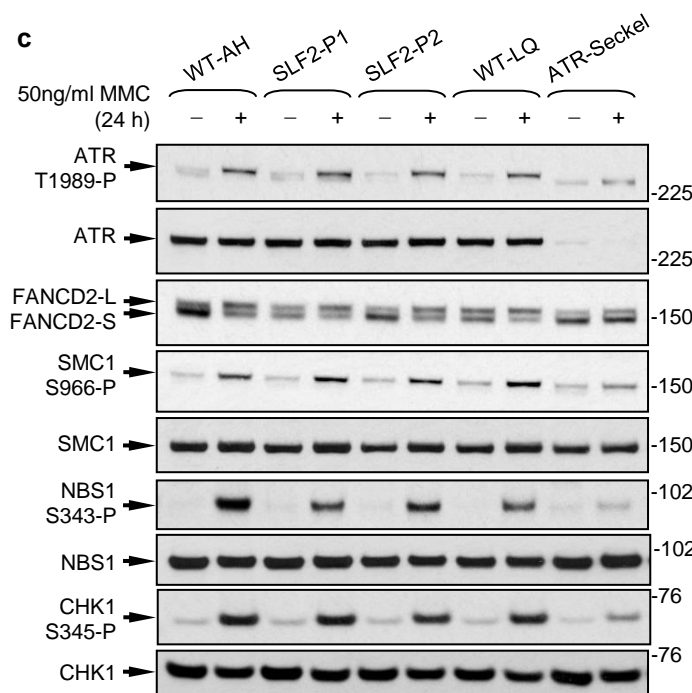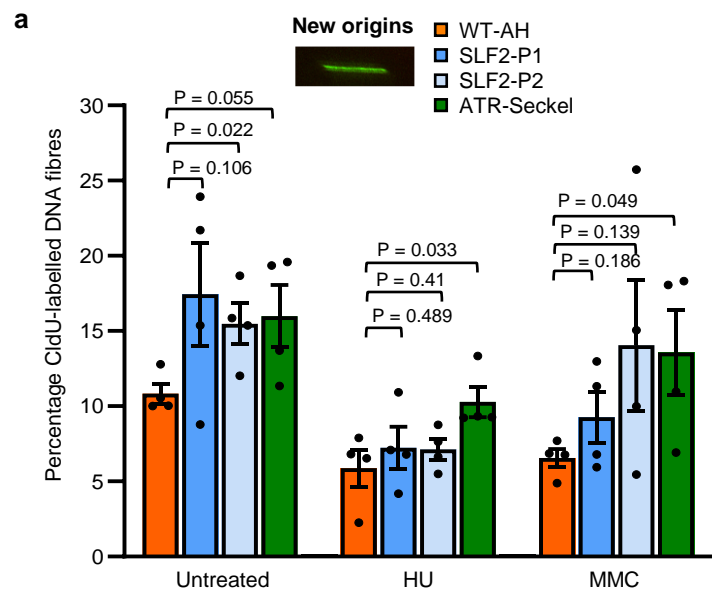
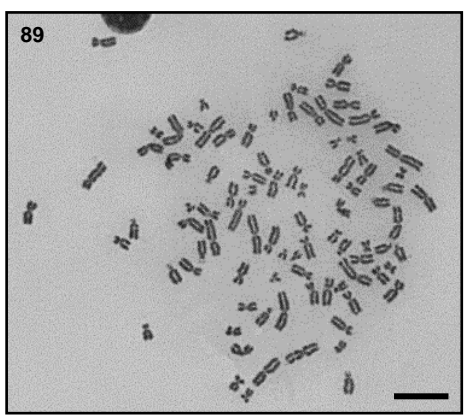# Supplementary Figure 15

Supplementary Figure 16
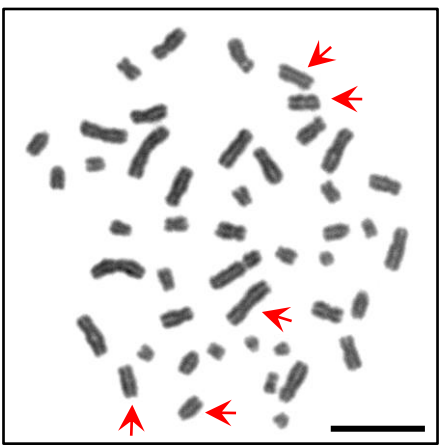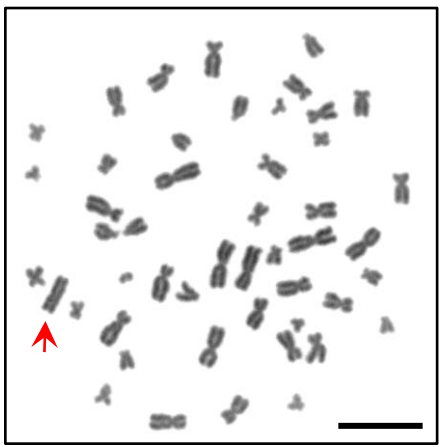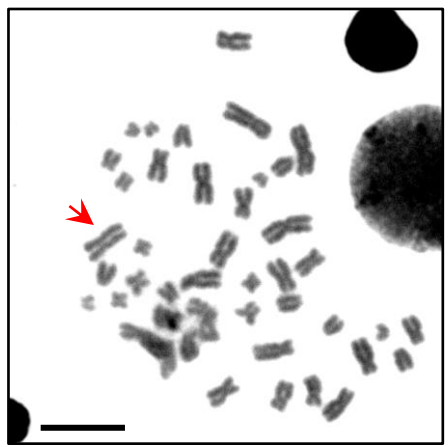
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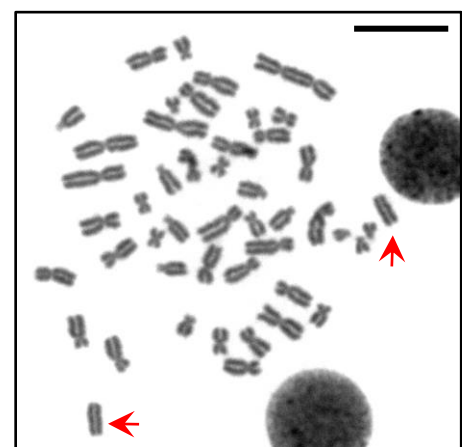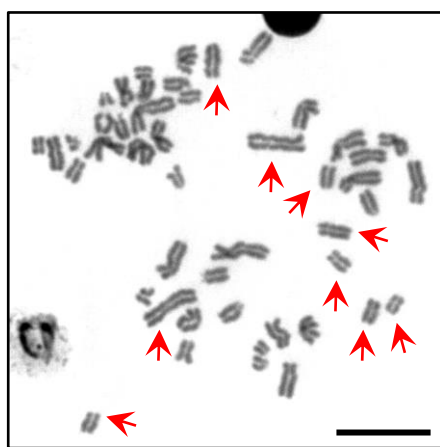
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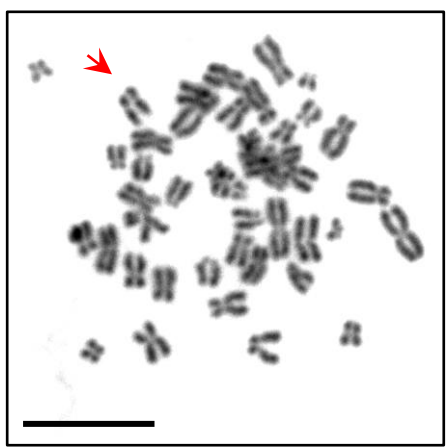Control   SLF2 patients
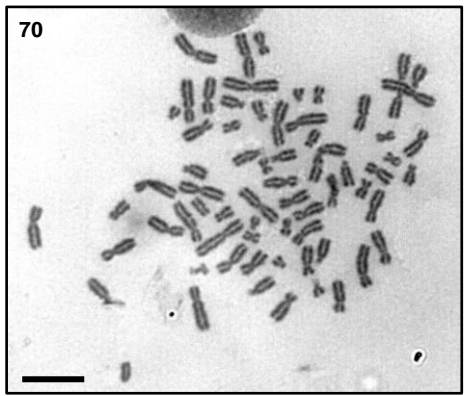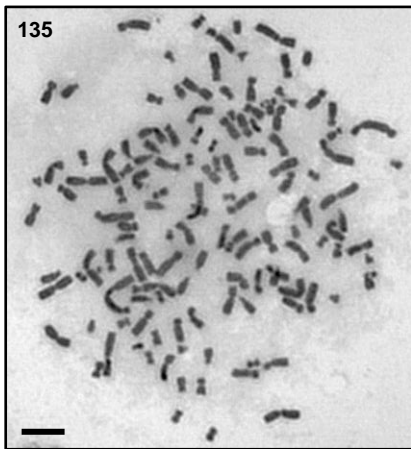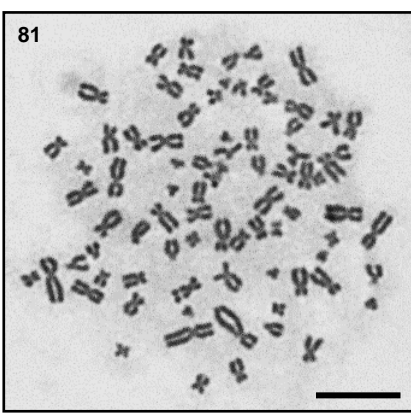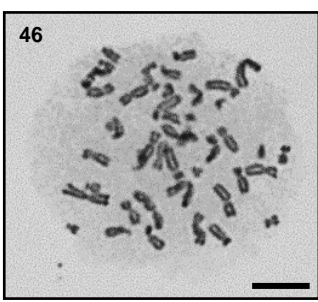
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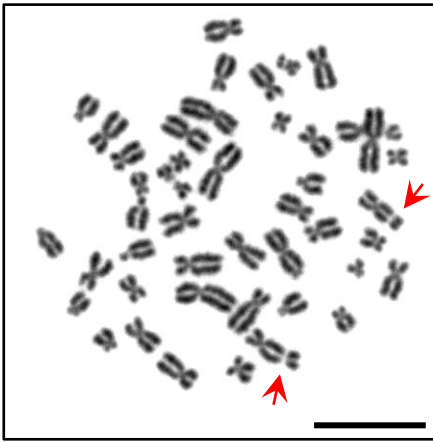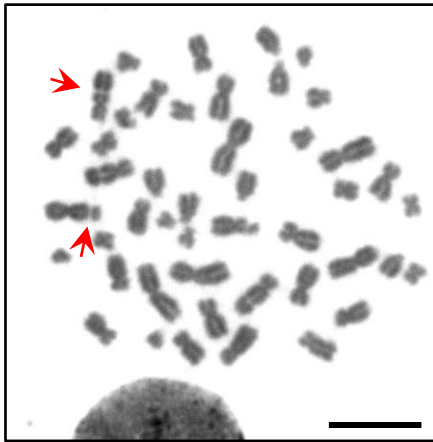SLF2-P3   SLF2-P3   SLF2-P3

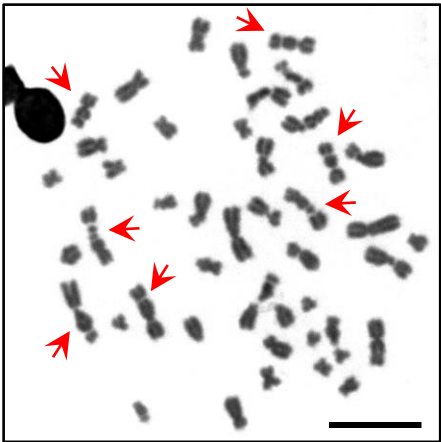SLF2-P1   SMC5-P7   SMC5-P7

Supplementary Figure 18

SMC5-P7

SLF2-P3

SLF2-P3

SLF2-P1

SLF2-P2

SLF2-P2

SMC5-P7
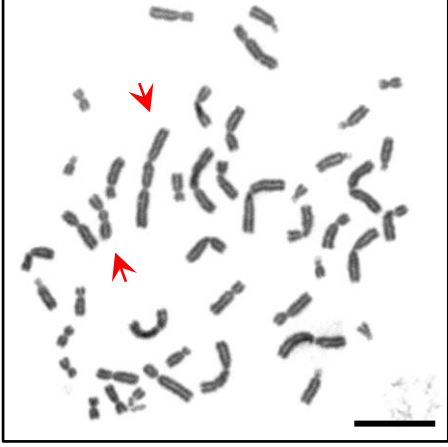
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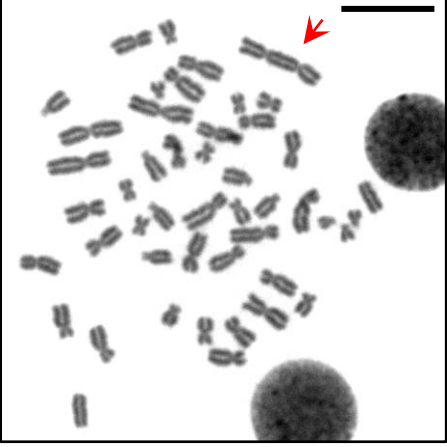
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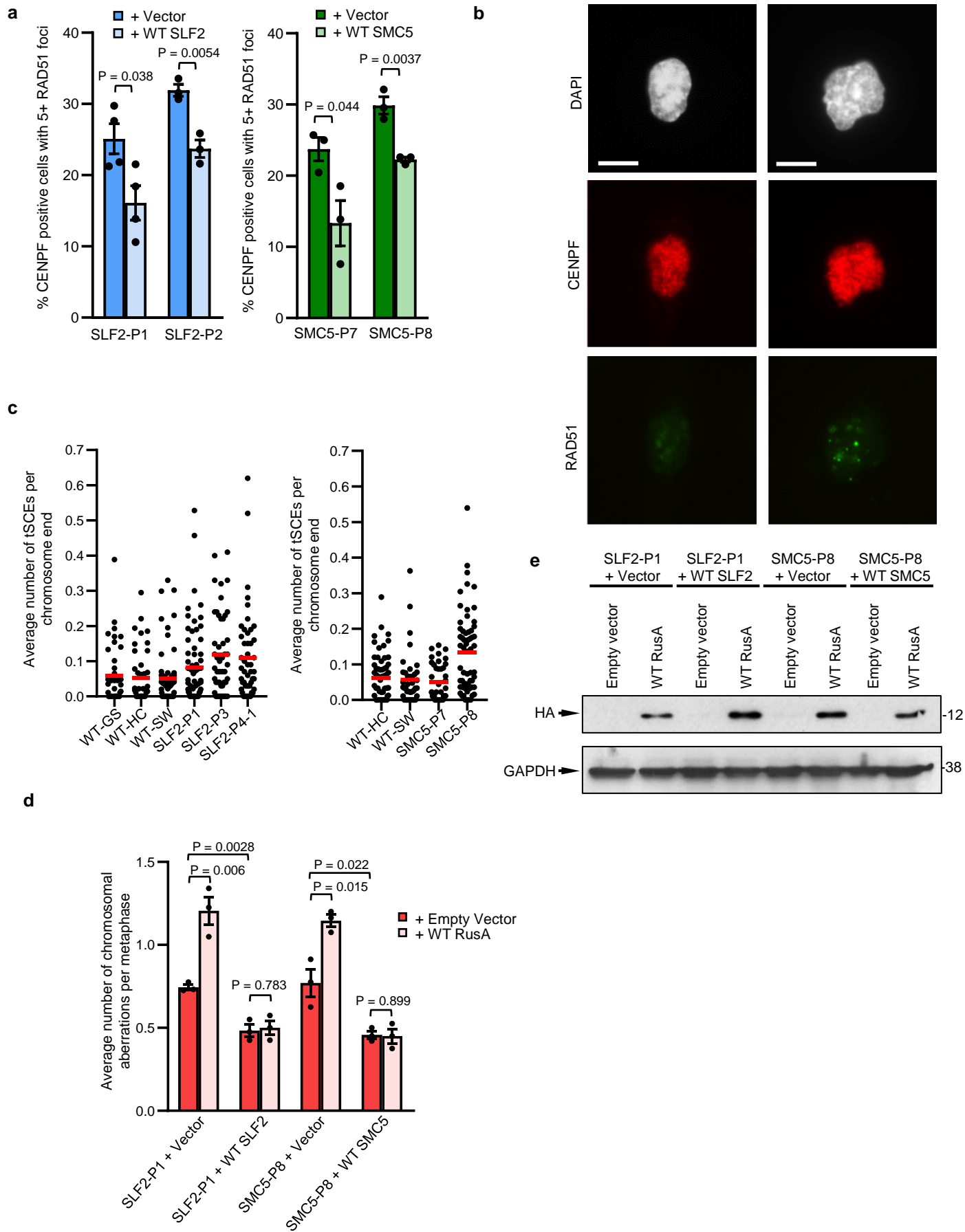
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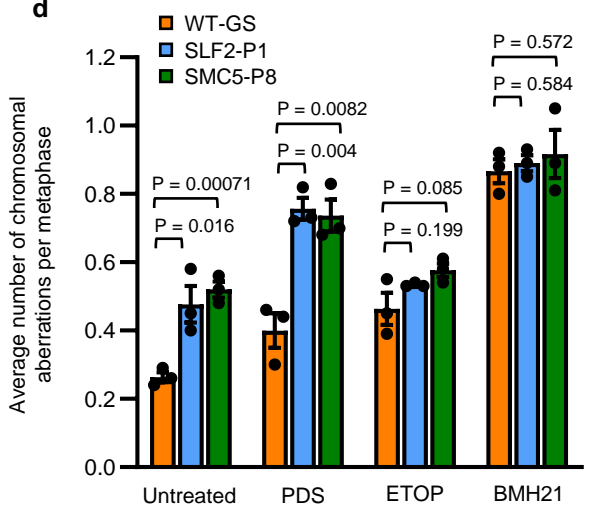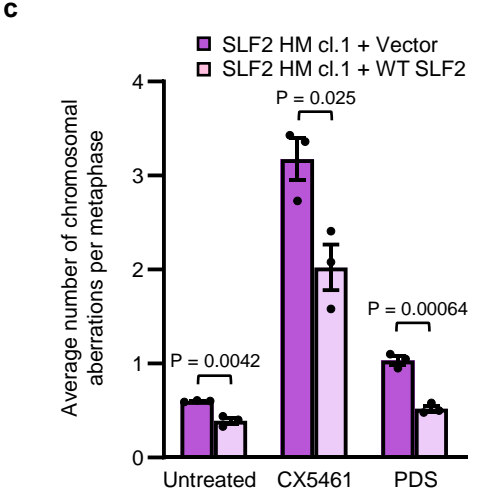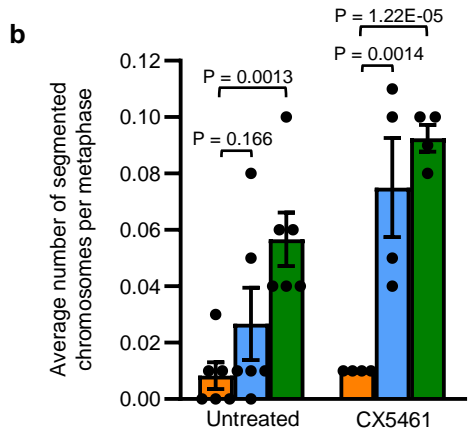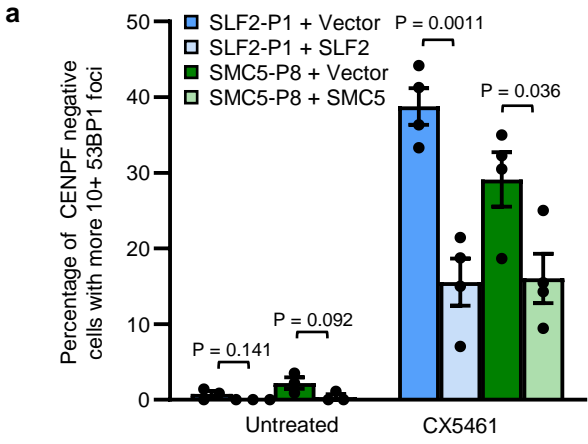
Supplementary Figure Legends

Supplementary Figure 1: Conservation of SLF2 and SMC5 amino acids mutated in patients

a Amino acid alignment of SLF2 protein from different species showing the degree of evolutionary conservation of disease causing SLF2 point variants, generated using Clustal Omega. Blue arrows indicate the missense variants present in SLF2-P2 (p.Gln1162His) and SLF2-P4-1 and SLF2-P4-2 (p.Asn861Ile). **b** Amino acid alignment of SMC5 protein from different species, generated using Clustal Omega, showing the degree of evolutionary conservation of the disease causing SMC5 point variants p.(Arg372del), present in SMC5-P7, and p.(His990Asp), present in SMC5-P8, SMC5-P9-1 and SMC5-P9-2. Blue arrows indicate location of the variants.

Supplementary Figure 2: Analysis of *SLF2* mRNA in SLF2-P2, SLF2-P3 and SLF2-P4-1

a PCR amplification of *SLF2* from cDNA derived from heathy normal WT individuals or SLF2 patients SLF2-P4-1 and SLF2-P3. **b** Chromatograms showing the skipping of exon 17 in the p.(Arg1110ArgΔexon17) variant from patient SLF2-P3. **c** A fragment of *SLF2* transcript (NM_018121.4) was amplified by RT-PCR from whole blood-derived mRNA from SLF2 patient SLF2-P2, as well as an age and sex matched control sample.

Supplementary Figure 3: Analysis of *SLF2* mRNA in SLF2-P2

a Top: Schematic of the two longest annotated *SLF2* transcripts, NM_018121.4 and NM_001136123.2 containing 20 and 19 exons, respectively. Black arrows indicate position of primers used for amplification of *SLF2*. Variant c.3486G>C, (p.Gln1162His; red arrow) affects the last nucleotide of NM_018121 exon 19 (splice donor). Bottom: Enlarged view of the 3' terminal regions of the NM_018121 and NM_001136123 transcripts. Variant c.3486G>C, (p.Gln1162His) is indicated as a red arrow. Blue arrowhead shows stop codon used by either NM_018121 or NM_001136123 transcripts. Red dashed lines indicate identical sequences between NM_018121 and NM_001136123 transcripts. **b** Bioinformatic predictions indicate disrupted splicing at the exon 19 donor site (NM_018121) by the c.3486G>C, (p.Gln1162His) variant. MaxEntScan, NNSPLICE, Human Splicing Finder have been used with Alamut software to examine the probability of splicing through a donor (top, delineated by red box, labelled 5') or acceptor paradigm (bottom, labelled 3'). Range of possible values is indicated. Values obtained with each tool are indicated as black boxes. Variant c.3486G>C, p.(Gln1162His), is indicated

in red. **c** RT-PCR on pooled cDNA from healthy human tissues using isoform-specific primers. Arrows show the expected size for NM_001136123 (primers P1 and P3), NM_018121 (primers P1 and P2) and β-actin. **d** RT-PCR of NM_018121 using cDNA derived from eight different adult tissues indicates ubiquitous expression using primers P1 and P2 (Supplementary Table S14). **e** RT-PCR on patient or control cDNA obtained from whole blood extracts indicates an isoform-specific splice defect leading to disruption of NM_018121. Primers specifically amplify NM_018121 (P6 and P8, T1, PCR3), NM_001136123 (P6 and P7, T2, PCR2) or both NM_018121 and NM_001136123 (P4 and P5, T1+T2, PCR1). Black arrows indicate result for transcript NM_018121, absent in the affected individual although present in control sample. **F** Representative immunoblot analysis of cell extracts from U-2 OS cells transiently transfected with constructs expressing either WT SLF2 or the patient associated SLF2 variant p.(Gln1162His) tagged with GFP. SLF2 constructs were mixed with an equal amount of mCherry expressing vector as a transfection control. Experiments in panels c, d, e and f are representative of three independent experiments with similar results.

Supplementary Figure 4: Analysis of RAD18-SLF1/2-SMC5/6 complex interactions and recruitment to DNA damage

a & **b** Co-immunoprecipitation of SLF2 deletion mutant interacting proteins with SLF1 and RAD18. (left) Schematic of SLF2 deletion mutants. (right) U-2 OS cells transfected with SLF2 deletion constructs were subject to HA-streptavidin pulldown (DP) and immunoblotted with the indicated antibodies to determine binding of GFP-SLF1 and RAD18. **c** Co-immunoprecipitation of SLF2 minimal binding region (MBR) interacting proteins. (left) Schematic of SLF2 MBR constructs. (right) U-2 OS cells transfected with SLF2 MBR constructs were subject to HA-streptavidin pulldown and immunoblotted with the indicated antibodies. **d** Co-immunoprecipitation of SLF2 deletion mutant interacting proteins as in (a) with SMC6 and RAD18. **e** Representative images of U-2 OS cells transiently transfected with WT or mutant GFP-SLF2 constructs after laser micro-irradiation. **f** Representative images of U-2 OS cells transiently transfected with WT or mutant GFP-SMC5 constructs after laser micro-irradiation. Note that nuclear GFP signal is lost from p.(Arg425Ter) with pre-extraction. Cells in (e & f) were recovered for 1 hour post irradiation and CSK pre-extracted prior to fixation, staining and imaging (scale bar = 20 µM). Experiments in panels a, b, c, d, e and f are representative of three independent experiments with similar results.

Supplementary Figure 5: Structural modelling of the SMC5 p.(Del372Arg) mutation.

(Left) Secondary structure model showing selected amino acid side chains from the X-ray crystal structure of a short section of *Saccharomyces cerevisiae* Smc5 (PDB: 3HTK) in complex with Nse2 (grey surface). An AlphaFold model for the relevant section of the Smc5 arm has been structurally superposed (AF-Q08204-F1) to extend the two helices towards the hinge (as this region is absent from the crystal structure). (Right) Secondary structure model showing the equivalent region from an AlphaFold model of human SMC5 (AF-Q8IY18-F1). Arg372 is located within a small, charged motif (369-RQRR-372) that sits near to a region of predicted disorder in the opposing helix; a similar motif can be found in budding yeast (365-RTKK-368). A hydrophobic residue (Ile744 and Leu728, in budding yeast and humans, respectively) serves to anchor the restarted (descending) helix. The directionality of each helix from the Smc5 arm (N- to C-terminus) is indicated by an arrow, heading either towards (ascending) or away (descending) from the hinge. Of note, the C-terminal tail of Nse2 emerges in close proximity to the predicted break in the descending helix.

Supplementary Figure 6: Structural modelling of the SMC5 p.(His990Asp) mutation.

a (top) Secondary structure models showing selected amino acid side chains from *Pyrococcus furiosus* RAD50 (*Pf*.Rad50) in un-liganded (left) and ATP-bound forms (right); PDB accession codes 1F2T and 1FTU respectively. The ATP-binding cassette (ABC) signature motif, containing Ser793, is additionally highlighted with carbon atoms coloured in yellow. The side chains of Phe791, Arg787 and Trp782 are repositioned as a result of ATP-binding and interaction with a second Rad50 monomer (not shown). **a** (bottom) Comparative view for the same region of human SMC5 (AlphaFold model, AF-Q8IY18-F1; UniProt entry SMC_HUMAN). Side chains for amino acids in equivalent positions to those shown in the top panel are shown in stick representation. His990 of human SMC5 is structurally equivalent to Phe791 of *Pf*.Rad50 (carbon atoms coloured green and orange respectively) but is also within hydrogen-bonding distance of the side chain of Thr987 (black dotted line). Mutation of His990 to Asp (p.His990Asp) is likely to be tolerated, without any gross effects on protein folding as no major steric clashes are predicted by the change in amino acid identity [Mutagenesis Wizard, PyMOL]. However, its introduction would affect the overall charge and electronics of the region accepting the adenine moiety of bound ATP. In addition the p.(His990Asp) mutation would affect stacking/packing interactions with the side chains of both

His984 and Phe977 (by analogy to *Pf*.Rad50). **b** Summary of prediction outcomes from the DynaMut webserver: http://biosig.unimelb.edu.au/dynamut. The SMC5 p.(His990Asp) patient mutation is predicted to generate only small increases or decreases in $\Delta\Delta G$ and thus no gross effect on the overall protein fold. A moderate decrease in molecule flexibility is predicted, but this is limited to just the loop containing the affected amino acid (see inset molecular model, region coloured in dark blue).

Supplementary Figure 7: Efficiency of reagents used to target *slf2* and *smc5* in zebrafish larvae.
a Schematic of the *Danio rerio slf2* genomic locus (GRCz11). Filled rectangles denote coding exons; black lines indicate introns. Target position of single guide RNAs (sgRNA) and morpholinos (MO) used are indicated with vertical arrows. **b** Polyacrylamide gel image showing heteroduplex analysis of PCR products amplified from genomic DNA harvested at 2 dpf from control embryos and embryos injected with *slf2* sgRNAs plus Cas9 protein. Embryos revealed high mosaicism of frameshifting insertions and deletions at each respective target site (*slf2* sgRNA1: 82%; *slf2* sgRNA2: 70%). Asterisks indicate homoduplexes of WT PCR product. **c** Representative sequences generated from PCR products cloned into TOPO-TA vectors. Plasmids were purified from individual colonies and subjected to direct sequencing, revealing insertions and deletions in *slf2* F0 mutant larvae. Protospacer adjacent motif (PAM) is shown with red dashed box. **d** Representative sequences confirming an 8 bp deletion in *slf2*$^{-/-}$ mutants. **e** qRT-PCR depicts 50% reduction in *slf2* mRNA level normalized to β-*actin*. F1-5' and F2-3' indicate two different primer sets complementary to the 5' and 3' regions of the *slf2* mRNA, respectively. n=2 independent experiments. **f** Schematic of the Danio *rerio smc5* genomic locus (GRCz11). Filled rectangles denote coding exons; black lines denote introns. Target position of single guide (sg) RNAs and MO used are indicated with vertical arrows. **g** Polyacrylamide gel image showing heteroduplex analysis of PCR products amplified from genomic DNA harvested at 2 dpf from control embryos and embryos injected with *smc5* sgRNA plus Cas9 protein. High mosaicism of frameshifting insertions and deletions at each respective target site is visible (*smc5* sgRNA1: 92%; *smc5* sgRNA2: 80%). Asterisks indicate homoduplexes of WT PCR product. **h** Representative sequences generated from PCR products (panel g) cloned into TOPO-TA vectors. Plasmids were purified from individual colonies and subjected to direct sequencing, revealing insertions and deletions in *smc5* F0 mutant larvae. Protospacer adjacent motif (PAM) is shown with red dashed box. Polyacrylamide gels in panels b and g were generated for screening purposes and so are representative of one experimental repeat.

Supplementary Figure 8: Loss of *slf2* and *smc5* in zebrafish give rise to microcephaly and aberrant craniofacial patterning

a Agarose gel images show exon exclusion of *slf2* exon 11 in morphants (MO) resulting in a 103 bp deletion (Δ) as determined by RT-PCR and sequencing. **b** Agarose gel images show semi-quantitative reduction of WT message in *smc5* MO as determined by RT-PCR. Agarose gels in b and c are representative of one experimental repeat. **c** RT-PCR product sequence confirmation of exon 11 skipping in MO as determined by cloning and sequencing of the lower *slf2* band in the morphant lane of (a). **d** RT-PCR sequence confirmation of exon 3 skipping in MO as determined by cloning and sequencing the *smc5* band in the morphant lane of (b). **e-h** Quantification of lateral head size (e & g) (left to right; 47, 42, 39, 37 embryos/condition were analysed from 3 independent experiments for panel e, and 27, 33, 20, 13 embryos/condition were analysed from 3 independent experiments for panel g), and ceratohyal angle measurements (f & h) (left to right; 19, 16, 24, 13 embryos/condition were analysed from 3 independent experiments for panel f, and 17, 16, 16, 24 embryos/condition were analysed from 3 independent experiments for panel h), of larvae injected with different doses (3 ng, 6 ng and 9 ng) of MO, or MO with co-injection of human WT *SLF2* or *SMC5* mRNA. Error bars represent standard deviation of the mean. Statistical differences were determined with an unpaired Student's t-test (two sided).

Supplementary Figure 9: Loss of *slf2* and *smc5* in zebrafish give rise to microcephaly and aberrant craniofacial patterning.

a Representative bright field lateral (top) and ventral images of the GFP signal in the anterior region of *-1.4col1a1:egfp* transgenic reporter larvae (bottom) showing controls, *slf2* morphants (MO) and *slf2* MO rescued with human WT *SLF2* mRNA, respectively. **b** & **c** Quantification of lateral head size (b) (left to right; 38, 34, 34 embryos/condition from 3 independent experiments) or ceratohyal angle measurements (c) (left to right; 27, 13, 22 embryos/condition from 3 independent experiments). **d** Representative bright field lateral (top) and ventral images of the GFP signal in the anterior region of *-1.4col1a1:egfp* transgenic reporter larvae (bottom) showing controls, *smc5* MO and *smc5* MO rescued with human WT *SMC5* mRNA, respectively. **e** & **f** Quantification of lateral head size (e) (left to right; 46, 45, 45 embryos/condition from 3 independent experiments) or ceratohyal angle measurements (f) (left

to right; 18, 24, 22 embryos/condition from 3 independent experiments). **g** Left, representative lateral bright field images; and right, representative ventral GFP signal showing in the mandible of -*1.4col1a1:egfp* transgenic reporter larvae at 3 dpf. Images show head size (left) and craniofacial patterning (right) in controls, in *smc5* MO, MO rescued with human SMC5 WT mRNA, and MO complemented with p.(His990Asp) patient variant. Left, white dashed shape depicts head size measured; right, white dashed lines show the ceratohyal angle. Abbreviations: MK, Meckel's cartilage; CH, ceratohyal cartilage (indicated with arrowheads, respectively); CB, ceratobranchial arches (asterisks); MO, morpholino. Scale bar, 300 μm, with equivalent sizing across panels. **h** & **i** Quantification of lateral head size (h) (left to right; 54, 36, 39, 33, 39, 40, 34 embryos/condition from 3 independent experiments) and ceratohyal angle measurements (i) (left to right; 34, 42, 49, 32, 33, 37, 21 embryos/condition from 3 independent experiments) of larvae injected with MO alone, co-injection of MO with human WT or variant encoding mRNA; p.(Arg733Gln) is a negative control (rs59648118; 16 homozygotes in gnomAD). For all panels: Statistical differences were determined with an unpaired Student's t-test (two sided). Error bars represent standard deviation of the mean. Scale bars, 300 μm with equivalent sizing across panels.

Supplementary Figure 10: *slf2* and *smc5* depletion induces apoptosis and altered cell cycle progression in zebrafish larvae.

a Representative dorsal inverted fluorescent images indicating TUNEL positive cells in *slf2* MO at 2 dpf. The blue dashed box indicates the region of interest (ROI). **b** Quantification of TUNEL positive cells in controls and larvae injected with *slf2* MO with or without WT mRNA (left to right; 35, 29, 28 embryos/condition from 3 independent experiments). ROI used is shown in panel (a). **c** Representative dorsal inverted fluorescent images indicating pHH3 positive cells in *slf2* MO at 2 dpf. **d** Quantification of pHH3 positive cells in larvae injected with *slf2* MO with or without WT mRNA (left to right; 24, 25, 25 embryos/condition from 3 independent experiments). ROI used was the same as that shown in panel (c). **e** Representative dorsal inverted fluorescent images show TUNEL positive cells in *smc5* MO at 3 dpf. **f** Quantification of TUNEL positive cells in controls and larvae injected with *smc5* MO with or without WT mRNA (left to right; 32, 31, 29 embryos/condition from 3 independent experiments). **g** Representative dorsal inverted fluorescent images indicating pHH3 positive cells in *smc5* MO at 2 dpf. **h** Quantification of pHH3 positive cells in controls and larvae injected with *smc5* MO and WT mRNA

(left to right; 24, 23, 24 embryos/condition from 3 independent experiments). In all cases, embryos of the same developmental stage and similar magnification were assessed for all *slf2* or *smc5* conditions. Fluorescent staining in the ROI was quantified using the ImageJ (NIH) ICTN plugin. Error bars represent standard deviation of the mean. Scale bar in panels a, c, e, g: 30 μm with equivalent sizing across panels. In all cases, statistical differences were determined with an unpaired Student's t-test (two sided).

Supplementary Figure 11: Replication fork analysis of SLF2 and SMC5 patient-derived cell lines

a Representative immunoblot analysis of myc-SLF2 expression in SLF2 patient fibroblasts infected with lentiviruses encoding myc-tagged WT SLF2 or an empty vector. A nonspecific cross-reactive protein was used as a loading control. **b** Representative immunoblot analysis of SMC5 expression in SMC5 fibroblasts infected with lentiviruses encoding WT SMC5 or an empty vector. A nonspecific cross-reactive protein was used as a loading control. Immunoblotting analysis in panels a and b are representative of two independent experiments with similar results. **c** & **d** Replication fork velocity of ongoing forks in WT cells, SLF2 patient LCLs (A) or SMC5 patient LCLs (B). n=3 independent experiments. A minimum of 430 fork structures were counted. Red lines denote median values. A Mann-Whitney rank sum test was performed for statistical analysis. **e** & **f** DNA fibre analysis in untreated cells and cells exposed to replication stress in SLF2 patient-derived LCLs (e) or SMC5 patient-derived LCLs (f) was carried out. In untreated cells, the indicated cell lines were pulse-labelled with CldU for 20 min, and then pulse-labelled with IdU, for 20 min. For DNA fibres following MMC treatment, cells were incubated with 50 ng/ml MMC for 24 h prior to pulse-labelling with CldU and IdU. For DNA fibres following hydroxyurea (HU) treatment, cells were pulsed with CldU for 20 min, exposed to 2 mM HU for 2 h and then pulsed with IdU for 20 min. The percentage of stalled forks was quantified. n=4 independent experiments. A minimum of 650 fork structures were counted. A Student's t-test (two-sided, equal variance) was performed for statistical analysis. Error bars denote SEM.

Supplementary Figure 12: Generation of U-2 OS SLF2 CRISPR hypomorphic cell lines

a Schematic of the human *SLF2* genomic locus. Filled rectangles indicate coding exons; black lines denote introns. Positions of single guide RNAs (sgRNA) are highlighted by red text and the location of the protospacer adjacent motif (PAM) is indicated by blue text. **b** Table detailing SLF2 variants present

in U-2 OS SLF2 CRISPR HM clones cl.1 and cl.2. **c** Representative immunoblot analysis of SLF2 expression in U-2 OS SLF2 CRISPR HM cell lines infected with lentiviruses encoding myc-tagged WT SLF2 or an empty vector. GAPDH was used as a loading control. **d** Representative immunoblot analysis of myc-SLF2 expression in U-2 OS SLF2 CRISPR HM cell lines infected with lentiviruses encoding myc-tagged WT SLF2 or an empty vector. Endogenous c-Myc was used as a loading control. Immunoblotting analysis in panels c and d are representative of two independent experiments with similar results.

Supplementary Figure 13: Analysis of the ATR-CHK1 dependent replication stress response in SLF2 and SMC5 patient-derived LCLs

a & **b** DNA fibre analysis of SLF2 (a) and SMC5 (b) patient-derived LCLs was carried out as in (Supplementary Figure 11 e & f) and the percentage of new origins (IdU only) were quantified. A representative image is included. n=4 independent experiments. A minimum of 650 fork structures were counted. A Student's t-test (two-sided, equal variance) was performed for statistical analysis. Error bars denote SEM. **c-f** Representative immunoblot analysis for the indicated proteins in whole-cell extracts from SLF2 (c & e) or SMC5 (d & f) patient-derived LCLs subjected to treatment with 0.5 mM HU for 2 h (c & d) or 50 ng/ml MMC for 24 h (e & f). In all cases, immunoblotting analysis are representative of two independent experiments with similar results.

Supplementary Figure 14: Representative microscopy images of SLF2/SMC5 mutant cell lines exhibiting elevated levels of S-phase associated DNA damage

a Representative immunofluorescence microscopy images of EdU positive S-phase cells with 53BP1 foci quantified in Figure 6a. **b** Representative immunofluorescence microscopy images of mitotic cells with MiDAS quantified in Figure 6b. **c** Representative immunofluorescence microscopy images of 53BP1 bodies in CENPF negative G1 cells quantified in Figure 6c. **d** Representative immunofluorescence microscopy images of cells with micronuclei quantified in Figure 6d. **e** Representative brightfield microscopy images of different types of chromosomal aberrations quantified in Figure 6f-l, Figure 8e, Supplementary Figure S15a-b, Supplementary Figure 19c, Supplementary Figure S20c-d. In all cases, scale bars = 10 µM.

Supplementary Figure 15: Genome instability in SLF2/SMC5 mutant cell lines is not exacerbated by exogenous replication stress

a & **b** Quantification of the average number of chromosomal aberrations (which includes chromatid/chromosome gaps, breaks, fragments and radials) in metaphase spreads from SLF2 (a) and SMC5 (b) patient derived LCLs before treatment or following exposure to 500 nM APH or 50 ng/ml MMC for 24 h. n=3 independent experiments. A minimum of 140 metaphases were counted. A Student's t-test (two-sided, equal variance) was performed for statistical analysis. Error bars denote SEM. **c** & **d** Quantification of the average numbers of sister chromatid exchanges in metaphase spreads from SLF2 (c) and SMC5 (d) patient derived LCLs treated as in (a & b). n=3 independent experiments. A minimum of 100 metaphases were counted. Red lines denote median values. A Mann-Whitney rank sum test was performed for statistical analysis.

Supplementary Figure 16: Levels of mosaic variegated hyperploidy in SLF2/SMC5 mutant LCLs

a Quantification of the number of chromosomes per metaphase in SLF2 patient-derived LCLs. n=3 independent experiments. A total of 300 metaphases were counted. **b** Quantification of the number of chromosomes per metaphase in SMC5 patient-derived LCLs. n=3 independent experiments. A total of 300 metaphases were counted. **c** Quantification of the average number of CENPA positive and CENPA negative micronuclei in SLF2 and SMC5 mutant fibroblast cell lines infected with lentiviruses encoding WT SLF2, WT SMC5, or an empty vector. n=3 independent experiments. A minimum of 185 micronuclei were counted. Student's t-test (two-sided, equal variance) was performed for statistical analysis. Error bars denote SEM. **d** Representative images of CENPA positive micronuclei**. e** Representative immunofluorescence microscopy images of mitotic cells from SLF2-P1 LCLs with multi-polar spindles quantified in Figure 7h. In all cases, scale bars = 10 µM.

Supplementary Figure 17: Representative microscopy images of SLF2 and SMC5 patient LCLs exhibiting mosaic variegated hyperploidy and sister chromatid cohesion defects

a Representative bright field microscopy images of metaphases exhibiting mosaic variegated hyperploidy derived from peripheral blood of SLF2 and SMC5 mutant patients. **b** Representative bright field microscopy images of metaphases displaying railroad chromosomes derived from peripheral blood of SLF2 and SMC5 mutant patients. In all cases, scale bars = 10 µM.

Supplementary Figure 18: SLF2 and SMC5 patient cells exhibit a unique chromosomal breakage phenotype.

Representative bright field microscopy images of metaphases displaying segmented chromosomes derived from peripheral blood of SLF2 and SMC5 mutant patients. In all cases, scale bars = 10 µM.

Supplementary Figure 19: SLF2 and SMC5 patient cells exhibit increased levels of recombination intermediates

a Quantification of the percentage of S/G2 (CENPF positive) cells with >5 RAD51 foci in SLF2 and SMC5 mutant fibroblast cell lines complemented with either WT SLF2, WT SMC5, or an empty vector. A minimum of 850 CENPF positive cells in total were counted over 3 independent experiments for SLF2-P2, SMC5-P7 and SMC5-P8, and 4 independent experiments for SLF2-P1. Error bars denote standard error of the mean. For statistical analysis, a Student's t-test (two-sided, equal variance) was performed. **b** Representative immunofluorescence microscopy images of cells from panel a. Scale bars = 10 µM. **c** The average number of telomeric SCEs (tSCEs) per chromosome end was quantified in WT, SLF2 and SMC5 patient-derived LCLs. The red line denotes the mean. n = 2 independent experiments. **d** Quantification of the level of chromosomal aberrations per metaphase (chromatid/chromosome gaps, breaks, fragments and chromosome radials) in complemented SLF2 and SMC5 mutant fibroblast cell lines infected with either an empty lentiviral expression vector, or a vector expressing WT RUSA. n=3 independent experiments. A minimum of 120 metaphases were counted. Error bars denote standard error of the mean. Student's t-test (two-sided, equal variance) was performed for statistical analysis. **e** Representative immunoblot analysis of HA-tagged RUSA expression in SLF2 and SMC5 mutant patient fibroblasts infected with lentiviruses encoding myc-tagged WT SLF2/SMC5 or an empty vector. GAPDH was used as a loading control.

Supplementary Figure 20: SLF2 and SMC5 mutant cells exhibit increased genome instability in the presence of G-quadruplex stabilising agents

a Quantification of the percentage of G1-phase (CENPF negative) cells with >10 53BP1 bodies in SLF2 and SMC5 mutant fibroblast cell lines expressing WT SLF2, WT SMC5, or an empty vector, with or without exposure to 250 nM CX5451 for 24 h. n=4 independent experiments. A minimum of 390 G1-

phase cells were counted. **b** Quantification of the average number of segmented chromosomes per metaphase in SLF2 and SMC5 patient-derived LCLs before or after exposure to 250 nM CX5461 for 24 h. n=6 independent experiments for untreated cells and n = 4 for CX5461 treated cells. A minimum of 350 metaphases were counted. **c** Quantification of the level of chromosomal aberrations (chromatid/chromosome gaps, breaks, fragments and chromosome radials) per metaphase in U-2 OS SLF2 CRISPR HM cell lines complemented with either WT SLF2 or an empty vector before or after exposure to 250 nM CX5461 or 1 μM pyridostatin (PDS) for 24 h. n=3 independent experiments. A minimum of 100 metaphases were counted. **d** Quantification of the average number of chromosomal aberrations (chromatid/chromosome gaps, breaks, fragments and radials) in metaphase spreads from SLF2 and SMC5 patient derived LCLs either left untreated or exposed to 1 μM PDS, 50 nM etoposide (ETOP) or 1 μM BMH21 for 24 h. n=3 independent experiments. A minimum of 100 metaphases were counted. In all cases, a Student's t-test (two-sided, equal variance) was performed for statistical analysis. Error bars denote SEM.

Supplementary Tables:

Table S11. Primers used for SLF2/SMC5 in vivo modelling studies.

Purpose	oligo name	Sequence
slf2 sgRNA1 CRISPR/Cas9	*slf2* sgRNA 1	5'-CAATATAGAAGAGCTGGAGG-3'
*slf2*sgRNA1 CRISPR/Cas9 efficiency	*slf2* sgRNA1 PCR primer F	5'-AAATACCCATTTTTGCCAACAG-3'
slf2 sgRNA1 CRISPR/Cas9 efficiency	*slf2* sgRNA1 PCR primer R	5'-AGGATGACAGTTTTGGCTTGTT-3'
slf2 sgRNA2 CRISPR/Cas9	*slf2* sgRNA 2	5'-TCTTATTCCAGCAGAGACCG-3'
*slf2*sgRNA2 CRISPR/Cas9 efficiency	*slf2* sgRNA2 PCR primer F	5'-TTCCTCACTCATCTCACAGACG-3'
slf2 sgRNA2 CRISPR/Cas9 efficiency	*slf2* sgRNA2 PCR primer R	5'-CCTGGACTAGTCATCGTGTTCA-3'
slf2 MO-induced suppression	*slf2* e11i11 sb MO	5'-ATGAGAAAGTGGCTGGTATTACCT-3'
slf2 e11i11 sb MO efficiency	*slf2* e11i11 PCR primer F	5'-ACAGTGAAAGTAAAGGGGAGGAC-3'
slf2 e11i11 sb MO efficiency	*slf2* e11i11 PCR primer R	5'-AAAAGACTGATGAACGATGCCC-3'
smc5 sgRNA1 CRISPR/Cas9	*smc5* sgRNA 1	5'-GTTGCAGGTTCACGATCGGA-3'
smc5 sgRNA1 CRISPR/Cas9 efficiency	*smc5* sgRNA1 PCR primer F	5'-TGTGCTGAACATCAACCAGAG-3'
smc5 sgRNA1 CRISPR/Cas9 efficiency	*smc5* sgRNA1 PCR primer R	5'-AAACAAACGACGCTTGCATA-3'
smc5 sgRNA2 CRISPR/Cas9	*smc5* sgRNA 2	5'-AAAACATCTGTCCTGGGCCG-3'
smc5 sgRNA2 CRISPR/Cas9 efficiency	*smc5* sgRNA2 PCR primer F	5'-CAGCACGTACGATCACTCTGA-3'
smc5 sgRNA2 CRISPR/Cas9 efficiency	*smc5* sgRNA2 PCR primer R	5'-GCCAGACACAGTGGATGTGA-3'
smc5 MO-induced suppression	*smc5* e3i3 sb MO	5'-TGTAAAAACACATACTTACAGCTCT-3'
smc5 e3i3 sb MO efficiency	*smc5* e3i3 PCR primer F	5'-CCGGACCCAAACTGAACAT-3'
smc5 e3i3 sb MO efficiency	*smc5* e3i3 PCR primer R	5'-TCCTCCACTGCCTTCTGACT-3'
SMC5 mutagenesis	*SMC5*-p.R733Q-F	TGAAGAGGAAGAGCAAAAAGCAAGTACCA
SMC5 mutagenesis	*SMC5*-p.R733Q-R	5'-TGGTACTTGCTTTTTGCTCTTCCTCTTCA-3'
SMC5 mutagenesis	*SMC5*-p.H990D-F	5'-GAATTAACTCCTCATGATCAAAGTGGAGGTGAA-3'
SMC5 mutagenesis	*SMC5*-p.H990D-R	5'-TTCACCTCCACTTTGATCATGAGGAGTTAATTC-3'
SMC5 mutagenesis	*SMC5*-p.R425*-F	5'-GAAATAATTGATAAGTGAAGAGAGAGGGAAACT-3'
SMC5 mutagenesis	*SMC5*-p.R425*-R	5'-AGTTTCCCTCTCTCTTCACTTATCAATTATTTC-3'
SMC5 mutagenesis	*SMC5*-p.R372-del-F	5'-TTGACCGACAGAGGATAGGTAATACCCGC-3'
SMC5 mutagenesis	*SMC5*-p.R372-del-R	5'-GCGGGTATTACCTATCCTCTGTCGGTCAA-3'
SMC5 construct sequencing	*SMC5*-seq1	5'-GCAAGAAGACGTCAACTCCA-3'
SMC5 construct sequencing	*SMC5*-seq2	5'-CGAGCAGATAAGGTTGGGTTT-3'
SMC5 construct sequencing	*SMC5*-seq3	5'-GGAATATGAAAATGTTCGTCAGG-3'
SMC5 construct sequencing	*SMC5*-seq4	5'-TGGACGATCATATTGTACGTTTT-3'
SMC5 construct sequencing	*SMC5*-seq5	5'-CAGCAGAAGAAAGTATGTGGTG-3'
SMC5 construct sequencing	*SMC5*-seq6	5'-ACAGTGATCTCTGAGAAGAACAAA-3
SMC5 construct sequencing	*SMC5*-seq7	5'-GCAGTGTGCTGGTGAAGTTG-3'
SLF2 construct sequencing	*SLF2*-seq1	5'-AGAAGTTGGGTGCGTGGTT-3'
SLF2 construct sequencing	*SLF2*-seq2	5'-TTTGGCTAAATATTTGGAGGCTA-3'

SLF2 construct sequencing	*SLF2*-seq3	5'-TTCCCATGAATCAGAAGAGGA-3'
SLF2 construct sequencing	*SLF2*-seq4	5'-CACTTGGAACACGGGAAAGT-3'
SLF2 construct sequencing	*SLF2*-seq5	5'-GAGCAGGAGGCTTTCCTGTA-3'
SLF2 construct sequencing	*SLF2*-seq6	5'-ATCATCCGAAACAGCCACTT-3'
SLF2 construct sequencing	*SLF2*-seq7	5'-ATGCCCAGACAGAGTTCAGG-3'
SLF2 construct sequencing	*SLF2*-seq8	5'-TTTCCTGCCATTTTCCATGT-3'
SLF2 construct sequencing	*SLF2*-seq9	5'-AGTAGGCCGACAGTTCTGGA-3'
actinb2-RT-PCR	*actinb2*_F	5'-CCACCATGTACCCTGGCATT-3'
actinb2-RT-PCR	*actinb2*_R	5'-GTCACCTTCACCGTTCCAGT-3'
slf2 mRNA expression	*slf2*-qPCR-F1	5'-TCTCCTGCAAAAGTCCAGTTC-3'
slf2 mRNA expression	*slf2*-qPCR-R1	5'-GCCTCTCAGGACTTCGTCTG-3'
slf2 mRNA expression	*slf2*-qPCR-F2	5'-ATGCGTCCTTCATCTCTGCT-3'
slf2 mRNA expression	*slf2*-qPCR-R2	5'-TCTCTGGGCTGAGGGTAAGA-3'
actinb2 mRNA expression	*actinb2*-qPCR-F	5'-TTGTTGGACGACCCAGACAT-3'
actinb2 mRNA expression	*actinb2*-qPCR-R	5'-TGAGGGTCAGGATACCTCTCTT-3'

Table S12. Primers used for sequencing of SLF2/SMC5

SLF2 Sequencing Primers	Primer name	Sequence
	SLF2-760F	5'-AAGGAGCAAATGGAGCAGAGAA-3'
	SLF2-1624F	5'-TGCGCTCAGAATATGGCACT-3'
	SLF2-2556F	5'-GTCTGATGTAGCAGCTGTGTT-3',
	SLF2-2961F	5'-TGAACTCTCCAGTCATCCCCA-3'
	SLF2-1768R	5'-GGCTTTATCTGAAGGTGCTGC-3'
	SLF2-2575R	5'-ACACAGCTGCTACATCAGACA-3'
	SLF2-3437R	5'-CTGGCGACCAAGTCTTTCAC-3'
SMC5 Sequencing Primers	**Primer name**	**Sequence**
	SMC5-300F	5'-ACCTGCTTTCATGGGACGAG-3'
	SMC5-975F	5'-AGAAAAGGCAACAGATATTAAGGAG-3'
	SMC5-1563F	5'-GGTTTTCCTCAAAGAGGTTCGTG-3'
	SMC5-1681F	5'-GTTTTCCTCAAAGAGGTTCGTG-3'
	SMC5-2188F	5'-GAGGAAGAGCGAAAAGCAAGT-3'
	SMC5-2322F	5'-TGCTTTTCGCTCTTCCTCTTCA-3'
	SMC5-2486F	5'-CCGCATCTTCACAACTCCGT-3'
	SMC5-687R	5'-GCATGAGGTCTCGAGCTGTTT-3'
	SMC5-1194R	5'-GGGCTGAAGATTCTCGCAGT-3'
	SMC5-1234R	5'-TTCTCCTCTGTCGGTCAAGC-3'
	SMC5-3178R	5'-TTTTGCAGGAGCTTTGGTGT-3'

Table S13. Primers used for the generation of SLF2 and SMC5 deletion/mutation constructs

SLF2 deletion constructs	Sequences of primer pairs
SLF2 Δ1	5'-TCCAGCACAGTGGCGGCCGCAACAGCTCCAGAAGCCTTAG-3' and 5'-CTAAGGCTTCTGGAGCTGTTGCGGCCGCCACTGTGCTGGA-3'
SLF2 Δ2	5'-AGAAGAATGATAGAGATCGAAATTCTGGCAATTCTGGCCA-3' and 5'-TGGCCAGAATTGCCAGAATTTCGATCTCTATCATTCTTCT-3'
SLF2 Δ3	5'-GTGATGTGTTGCGCTTAGAAAACCTAGACAGTGATGAGGA-3' and 5'-TCCTCATCACTGTCTAGGTTTTCTAAGCGCAACACATCAC-3'
SLF2 Δ4	5'-CAGGAAATTCCAATGCAGGTCTGTTTCGGATGATGTCAGT-3' and 5'-ACTGACATCATCCGAAACAGACCTGCATTGGAATTTCCTG-3'
SLF2 Δ5	5'-AGATTTTTTTGACAACACAAAGGCAACTGAGACAGTGCCT-3' and 5'-AGGCACTGTCTCAGTTGCCTTTGTGTTGTCAAAAAAATCT-3'
SLF2 Δ6:	5'-TGGGCATAAATGAACTCTCCTAGGGGCCCGTTTAAACCCG-3' and 5'-CGGGTTTAAACGGGCCCCTAGGAGAGTTCATTTATGCCCA-3'
SLF2 Δ7	5'-GCCGAGGCATTAAATCCCCACCTGTCCCTGTGTTAAAGTG-3' and 5'-CACTTTAACACAGGGACAGGTGGGGATTTAATGCCTCGGC-3'
SLF2 Δ8	5'-CGTCTGCTTATCACTATGTCCCAATTTTTTCAACACTTCC-3' and 5'-GGAAGTGTTGAAAAAATTGGGACATAGTGATAAGCAGACG-3'
SLF2 Δ9	5'-GGAAAGAAAGTGAAGATTCACAGCTGGTCCCTAATTGGAC-3' and 5'-GTCCAATTAGGGACCAGCTGTGAATCTTCACTTTCTTTCC-3'
SLF2 Δ10	5'-ACAACCTCCTGTGGTTGGTATGTTCTCATTCTTTTTCTTC-3' and 5'-GAAGAAAAAGAATGAGAACATACCAACCACAGGAGGTTGT-3'
SLF2 Δ11	5'-CACTGAAAAGAAAACTAAGGTCCCCAATCAGAATTGGAGA-3' and 5'-TCTCCAATTCTGATTGGGGACCTTAGTTTTCTTTTCAGTG-3'.
MBR1	5'-GAACATGCGGCCGCTTCAATCAGTATACCTTG-3' and 5'-CGCTCTAGAGCCTAACTAACTTCACCGACTAA-3'
MBR2	5'-GAACATGCGGCCGCTTCAATCAGTATACCTTG-3' and 5'-CAGCATTCTAGACGCTAAGAATCTGGTACCCA-3'.

SLF2 and SMC5 mutant constructs	Sequences of primer pairs
SLF2 p.Ser815Ter	5'-TGTTTCGGATGATGTGAGTTCATACAGACTG-3' and 5'-CAGTCTGTATGAACTCACATCATCCGAAACA-3'
SLF2 p.Arg336Lysfs:	5'-AATTCCCTGAAAAAAAGAAAAAGGAACTCTG-3' and 5'-CAGAGTTCCTTTTTCTTTTTTTCAGGGAATT-3'
SLF2 p.ΔSer907Phefs	5'-TCCTGAAACCAACATTTTAAATG-3' and 5'-AAAAAATTGGCTTATAAGATGAATC—3'
SLF2 p.Asn861Ile	5'-GTGTTTTTCATTATGGGGATTGATTTTAG-3' and 5'-AGCTGCTACATCAGACAATG-3'
SLF2 .ΔAla1085_Arg1110	5'-AAACACTTTGTGCTACTC-3' and 5'-CTGCTTTTCAAGTTCTAAATG-3'
SLF2 p.Aps783Serfs	5'-TCAGATTTTTTTGACAACAC-3' and 5'-TGTTTTTCCCGATTTAAGAATAAG-3'
SMC5 p.Arg425Ter	5'-AATTGATAAGGGAAGAGAGAGG-3' and 5'-ATTTCGCCTTCACATAATG-3'
SMC5 p.ΔArg372	5'-GAGAATAGGTAATACCCGC-3' and 5'-TGTCGGTCAAGCTCTTCA-3'
SMC5 p.His990Asp	5'-AACTCCTCATGATCAAAGTGG-3' and 5'-AATTCATGCAGTTGAGTAC-3'

Table S14. Primers used for RT-PCR expression studies

Experiment	Primer name	Identifier	Sequence
Expression studies			
RT-PCR	SLF2_ex16_common_F	P1	GTGCAGATGAAGCCTTCTGA
RT-PCR	SLF2_1173_ex20_R	P2	GGTACCCAGAAGTCATGAAGC
RT-PCR	SLF2_1186_ex19_R	P3	TGAAGAGTGCCATTCAGCAA
RT-PCR	SLF2-E1-FOR	P4	CGCGCTGCCATCTGAGACCC
RT-PCR	SLF2-E3-REV	P5	GGACAGGCTGCTCCTGCTGC
RT-PCR	SLF2-E14-FOR	P6	GGACAGGCTGCTCCTGCTGC
RT-PCR	SLF2-I19-REV	P7	GGTGCCTGAACTCTGTCTGGGC
RT-PCR	SLF2-E20-REV	P8	TGAAGAGTGCCATTCAGCAAAACT