

Age-related remodelling of oesophageal epithelia by mutated cancer drivers

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SUMMARY

Clonal expansion in aged normal tissues has been implicated in cancer development. However, its chronology and risk-dependence are poorly understood. Here through intensive sequencing of 682 micro-scale oesophageal samples, we show progressive age-related expansion of clones carrying mutations in *NOTCH1*-predominant drivers in physiologically normal oesophageal epithelia (PNE), which is substantially accelerated by alcohol drinking and smoking. Driver-mutated clones emerge multifocally from early childhood and accompanying their own phylogenetic structure, increase their number and size with aging, ultimately replacing almost entire oesophageal epithelia in the extreme elderly. Compared with oesophageal cancer, there is a marked overrepresentation of *NOTCH1* and *PPM1D* mutations in PNE, which are typically acquired as the first hit before late adolescence, as early as early infancy, and significantly increase their numbers with heavy smoking and drinking. Remodelling of oesophageal epithelia by driver-mutated clones is an inevitable consequence of normal aging, impacting cancer development depending on lifestyle risks.

Introduction

Cancer is thought to comprise a heterogeneous population of neoplastic cells showing a complex hierarchical structure in terms of gene mutations¹⁻³. According to recent studies⁴⁻⁶, the entire cancer hierarchy by itself might be further embedded in higher level hierarchies that are recursively generated by multiple rounds of positive selections, where acquisition of driver mutations plays a central role⁷. Thus, before a cancer develops, many independent, precancerous populations of clones are presumed to be present within apparently or physiologically normal tissues⁸. However, it is poorly understood how those clones evolve from early infancy to the end of the life span in terms of their frequency and size and how its dynamics is affected by environmental/genetic factors to contribute to cancer. Major challenges to clarify these issues include rarity of such events, small size of clones, and more importantly, difficulty to retrospectively analyse those earlier clones.

Oesophageal squamous cell carcinoma (ESCC) represents the most common oesophageal cancer among Asian populations⁹, in which the roles of heavy alcohol drinking and smoking have been demonstrated^{10,11}; individuals having these lifestyle risks are associated with a substantially increased incidence of developing ESCC with a hazard ratio of as high as 6.12¹² and often develop multiple cancers or a second (metachronous) cancer after treatment of the primary tumour¹³. Moreover, in these high-risk individuals, *TP53*-mutated cells have been documented in physiologically normal oesophageal epithelia (PNE)^{13,14}, suggesting the presence of many precancerous foci before or at the onset of a cancer ("field effects¹⁵"). In this study, we investigated early clonal events in the oesophagus using multiple collection of micro-scale PNE samples, as small as 0.2 mm² in size, from individuals with varying age and lifestyle ESCC risk, followed by an unbiased detection of somatic mutations and copy number abnormalities (CNAs) using whole-exome sequencing (WES).

Somatic mutations in PNE tissues

To assess clonal expansion within PNE, we first performed WES of DNA from 5 mm² PNE samples (n=25), as well as cancer tissues (n=2), collected from a surgically resected oesophagus (**Extended Figs. 1 and 2a**). Somatic mutations were detected in most PNE samples (24/25) (**Extended Fig. 2b-d**). In contrast to abundant mutations shared between cancer samples, none of the mutations were shared among PNE samples mutually separated 10–15 mm apart, while small numbers of shared mutations were observed among closely (by 1.5 mm) positioned samples (**Extended Fig. 2c,d**). These results suggest pervasive expansions of multiple independent clones within the ESCC-bearing oesophagus.

To confirm the finding in the exemplary case and extend the observation to the oesophagus in healthy individuals, we collected a total of 157 PNE samples with varying sampling size, 0.2 mm² (n=53), 0.8 mm² (n=31), or 4 mm² (n=73), by punch biopsy from 130 endoscopically obtained specimens from ESCC/dysplasia patients (n=21) and healthy individuals (n=40) with or without lifestyle ESCC risk (**Extended Figs. 1a and 2e-g**). Samples were also collected from cancer (n=20) and dysplastic (n=12) tissues. Multi-regional sampling was performed in 16 individuals with a median of 5 (range: 2–8) samples per case. WES detected a total of 7,930 somatic mutations in 182 of 189 samples (**Extended Fig. 3a**). True positive rate in randomly selected 622 variants from 105 samples was 95.7% (**Online Methods**). Mutations were detected in all PNE and cancer samples from ESCC patients, the majority of whom had a long history of heavy drinking and smoking. Moreover, mutations were also detected in most samples from healthy individuals (99/106). None of the mutations were shared across different PNE samples from multi-regional biopsies, except for mutually closely positioned samples in two cases (UPN49 and UPN80) (**Extended Fig. 3b-j**). The number of detected mutations was affected by sample size. However, when sample size was stratified, mutation number linearly increased with subject's age, which was significantly accelerated by the presence of lifestyle ESCC risk (**Fig. 1a**). Uniformly decreasing with sample size, mutant cell fraction (MCF) of the largest clone (maxMCF) within a sample also increased with subject's age but was not significantly affected by lifestyle ESCC risk (**Fig. 1b**).

Signatures of mutations were analysed to decipher the underlying mechanism of mutational processes. Combining mutational data sets from 5 published studies¹⁶⁻²⁰,

we analysed a total of 54,121 single nucleotide variants (SNVs) in 157 PNE, 12 dysplasia, and 519 ESCC samples and identified 4 mutational signatures (designated as Sigs. A–D) that are predominant in ESCC and PNE (**Fig. 1c and Extended Fig. 4a**). Sig. A is thought to be an APOBEC-related signature and highly enriched in mutations in cancer samples compared with those from PNE and dysplastic tissues (**Fig. 1d**). Although not remarkable in PNE samples as a whole, Sig. A was more prominent in high-risk than low-risk samples (**Extended Fig. 4b,c**). Corresponding to age-related COSMIC signature 1 (Sig. 1), Sig. B was seen across all samples and represented the major source of mutations in low-risk PNE and cancer samples (**Fig. 1d and Extended Fig. 4a**). Highly enriched in high-risk PNE samples and dysplastic tissues (**Fig. 1d**), Sig. D is mainly composed of T>C transitions at ApTpN sites with a conspicuous transcriptional strand bias (**Fig. 1c**) and conformed to COSMIC signature 16 (Sig. 16)²¹. Accentuated in liver cancers in Japanese males²², Sig. 16 has recently been related to alcohol consumption on the basis of its association with alcohol drinking and two ESCC risk alleles involved in alcohol metabolism, *ALDH2* (rs671) (A) and *ADH1B* (rs1229984) (G), among Asian drinkers with ESCC¹⁸. The association with *ALDH2* was also confirmed in our ESCC cohort and TCGA stomach and hepatic cancers (**Extended Fig. 4d**). Although resembling COSMIC signature 4, the origin of this unique signature in oesophageal epithelium remains to be elucidated (**Extended Fig. 4e**).

Mutations in single cells in PNE

To obtain more insight into the mutations in PNE, we performed whole genome sequencing of 13 single PNE cell-derived colonies isolated from 7 individuals (**Extended Fig. 1b**). In total, we detected 26,901 somatic variants, which were largely explained by Sigs. A–D (**Fig. 2a,b and Extended Fig. 5**). One or more driver mutations were detected in 7 colonies. The mutation number in colonies from low-risk individuals linearly increased with age with an annual increase of 41.5 mutations/genome/year ($R^2=0.926$) or 0.53 mutations/exome/year ($R^2=0.840$) (**Fig. 2c,d**), which was comparable to those reported in intestinal mucosa and hepatic cells²³. Accumulation of mutations was accelerated by the lifestyle ESCC risk for the entire genome (51.9 mutations/genome/year), which was more prominent for exome regions (0.81 mutations/exome/year) due to a large contribution from transcription-coupled signature (Sig. D) (also see below). Unlike colon and small intestine, contribution of

age-related signature (Sig. B) is not conspicuous. Instead, Sig. A and Sig. C are the major contributors to mutations in all cells, while no clear linearity was seen for Sig. A (**Fig. 2e**). Prominently, SNVs in colonies from high-risk individuals were disproportionally dominated by Sig. D (Sig. 16). Except for Sig. A, the number of mutations in each signature linearly increased with age. However, unlike Sig. B and Sig. C components, which exhibited an excellent linearity regardless of lifestyle ESCC risk, Sig. D mutations in cells from high-risk individuals showed a large deviation from the linearity, further supporting the link between Sig. D and alcohol consumption.

Driver mutations in PNE samples

Significantly mutated genes, or drivers, among 157 PNE samples were interrogated by globally gauging recurrent involvement of nonsynonymous mutations across the entire genome²⁴ and also by locally assessing evidence for positive selection relying on dN/dS⁷. Both measures provided almost identical lists of driver genes, which only partly (6/10 genes) overlapped to those calculated for ESCC in a similar manner using combined WES data from 519 ESCC cases from 5 published studies¹⁶⁻²⁰ (**Fig. 3a**), suggesting substantial difference of major drivers between PNE and cancer. To further interrogate this, we re-evaluated for mutations 24 genes that were significantly mutated in either PNE or cancer in the original 157 PNE samples with higher sensitivity using targeted deep sequencing (median 1,077×) (**Fig. 3a**). Overall, mutations were found in all 24 drivers in PNE samples with similar mutational distributions (**Fig. 3a,b and Extended Fig. 6a**). However, mutation frequency of several genes substantially differed between PNE and cancer samples, suggesting discrete mechanism of positive selection in PNE and ESCC. Mutated in most cancer (414/519, 79.8%) and dysplasia (11/12, 91.7%) samples, *TP53* mutations were much less common (48/157, 30.6%) in PNE. Also, *NFE2L2*, *CDKN2A*, and *FBXW7* were less frequently mutated in PNE than cancer samples. By contrast, most frequently mutated in PNE samples was *NOTCH1* and other *NOTCH* family genes (104/157, 66.2%), which showed an impressive contrast to a disproportionately low *NOTCH1* mutation frequency in cancer samples (78/519, 15.0%).

Another gene differentially mutated between cancer and PNE was *PPM1D* encoding a TP53-induced phosphatase. Negatively regulating MAPK/p38, PPM1D inhibits phosphorylation of TP53, through which downregulating TP53-mediated

transcription and apoptosis²⁵. Although rarely seen in ESCC (0.58%), *PPM1D* mutations were found in a substantial fraction of PNE samples (12.7%). Other genes more frequently mutated in PNE than in ESCC included *NOTCH2*, *ZFP36L2*, *FAT1*, *NOTCH3*, *CHEK2*, and *PAX9*. The differential frequencies of driver mutations between cancer and PNE samples were largely recapitulated by targeted-capture sequencing of matched cancer/PNE samples from an additional 68 ESCC patients (**Extended Fig. 6b**).

Most of the high-risk PNE samples (62/64, 97%) harboured one or more driver mutations, as exemplified in the samples from a single oesophagus (UPN32) (**Extended Fig. 2c,d**). By contrast, low-risk PNE samples, particularly 0.2 mm² samples from younger individuals, frequently lacked known driver mutations; the positivity of driver mutations was 74% (69/93) of all low-risk samples. The impact of subject's age on the number of driver mutations and their clone size was evident (**Extended Fig. 6c,d**). Lifestyle risk also affected the number of driver mutations, even after the effect of age was adjusted (**Extended Fig. 6c,e,f**). The effect of lifestyle ESCC risk on mutation frequency was most prominent for *NOTCH1* mutations, followed by mutations in *PPM1D*, *EP300*, *TP53*, and *NOTCH2* (**Fig. 3c and Extended Fig. 6f**). Lifestyle risk also affected maxMCF, although the effect was less prominent for individual mutations (**Extended Fig. 6d,g,h**). Multiple mutations affecting the same gene, particularly those affecting *NOTCH1* and *TP53*, were frequently detected samples from high-risk individuals aged >50 years (45/54), which was rarely seen in samples from younger individuals (**Fig. 3d**).

Copy number abnormalities in PNE

CNAs were also seen in all dysplastic tissues but less extensive (**Extended Fig. 7a,b**). In PNE samples, CNAs were rare as a whole, but their frequency increased with age and slightly enhanced by exposure to lifestyle ESCC risk (**Extended Fig. 7c**). CNAs in PNE were largely confined to a small number of selected chromosomes and mostly explained by 9q UPD (**Extended Fig. 7a,d**). Accounting for 77% (49/64) of CNA-positive PNE samples, 9q UPD was more frequent in PNE from elderly individuals, particularly those with lifestyle ESCC risk (**Extended Fig. 7e**). In PNE, all 9q UPDs affected the *NOTCH1* locus in common and were invariably accompanied by *NOTCH1* mutations, suggesting that mutated *NOTCH1* is the target of 9q UPD/LOH (**Extended Fig. 7d**). 9q LOH was rather more frequent in cancer and dysplasia samples (48/57).

However, in these samples, 9q LOH was more frequently caused by deletion than UPD and often extended to the 9p arm, and the majority (40/57) lacked *NOTCH1* mutations, suggesting the presence of other targets than *NOTCH1*, such as *CDKN2A* (**Extended Fig. 7d**). Other recurrent CNAs in PNE included 3q gain (8.5%) and 17p LOH (7.4%), of which 17p LOH represents a major mechanism of bi-allelic inactivation of *TP53* in ESCC as well as other cancer types (**Extended Fig. 7f,g**).

Structure and history of clones in PNE

Next, to investigate clonal architecture in PNE, we performed high-density collection of 0.2 mm²-scale PNE samples (containing ~10,000 cells; **Extended Fig. 2g**) from 16 single biopsy specimens with a median of 22 (range: 8-34) sample/biopsy, followed by WES or targeted-capture sequencing of 24 ESCC/PNE drivers (**Fig. 4 and Extended Figs. 1c,8,9**). Analysis of densely collected micro-scale samples allowed for deciphering spatial distribution and mutational composition of clones more precisely than that of solitary samples (**Extended Fig. 9**).

In the three specimens taken from low-risk individuals in 20's (**Fig. 4b and Extended Figs. 8b-d and 9a,b**), small numbers of mutations having small MCFs were shared by discrete sets of samples. Carrying much higher numbers of private mutations than the shared mutations, samples in each set were observed over an epithelial area as large as 18.1 mm² in size, spatially interrupted by other samples with which no mutations were mutually shared. These findings suggest that during early development to early adult life, multiple stem cells and their progenies in the rapidly enlarging oesophagus spread over a nearby region, being intermingled with each other, and after development, locally replenishing the oesophageal epithelium they resided in. Thus, the small number of mutations (median 1 [0–6], equivalent to 1.9 [0–11.3] years), which are acquired before spreading, are thought to represent somatic mosaicism rather than positive selection. Driver mutations were found only in a fraction of samples with small MCFs and rarely involved ≥ 2 samples (**Fig. 4b and Extended Fig. 9a,b**), suggesting that a limited number of driver-mutated clones expanded to the detectable size.

PNE in older individuals, by contrast, had substantially different clonal architectures, as exemplified in five biopsies from three high-risk (UPN39, UPN134, and UPN135) and two low-risk (UPN55 and UPN59) individuals aged 70 years or more

(**Fig. 4c and Extended Figs. 8e,9f,g**). Shared mutations markedly increased in their number and MCF. Compared with mosaic mutations, they were more likely segregated into discrete sets of ≥ 2 samples (median 3, range [2-10]), which were tightly clustered together and keeping close proximity to each other, occupied a relatively small region no larger than 12.8 (median 2.87: range 0.79–12.8) mm² (**Fig. 4a**).

Combining the results from all 16 biopsies, density of expanded driver-mutated clones and their average size increased with age, which was enhanced by exposure to lifestyle ESCC risk, minimally affecting their average clone size (**Fig. 5a**). Although accounting only for $\leq 20\%$ of all epithelial areas before 50's, driver mutated clones increased their number and size after 50's and until at the age of 70, replaced a substantial fraction of the entire oesophageal epithelium (**Fig. 5b**). High-risk elderly individuals exhibited a higher mutation density (≥ 50 years of age: 7.5-103.8/cm²) and a larger size of *TP53*-mutated clones, compared with those from low-risk younger individuals (< 50 years of age: 0-18.3/cm²) (**Fig. 5c**). Clones carrying biallelic *TP53* lesions were exclusively seen in biopsies from high-risk individuals. In accordance with the observation in solitary sampling, a higher *NOTCH1* mutation density was observed in biopsies from high-risk elderly individuals (35.1-173.1/cm²) than low-risk younger ones (0-39.5/cm²), particularly for biallelic mutations (**Fig. 5c**). *PPM1D* mutations also tended to be more frequent in high-risk elderly individuals.

Unbiased detection of mutations in densely collected micro-scale samples also allowed us to illustrate a complexity of evolutionary history of expanded clones, which is inseparably linked to the geographic substructure of PNE (**Fig. 6a and Extended Fig. 10**). In a representative PNE biopsy sample obtained from an 81-year-old high-risk individual, divergent clones populating a small epithelial region (~ 1.8 mm²) were originated from a single stem cell that had acquired a *NOTCH1* mutation almost 80 years before, when the subject was $< \sim 1.9$ years of age (**Fig. 6a, left panel**). Through a series of recursive positive selections over ~ 80 years, four additional driver mutations were fixed in the different subpopulations in varying combinations. In another example, clonal evolution was initiated by a stem cell in which a *TP53* mutation occurred at the age of ~ 13.2 years (**Fig. 6a, right panel**). Acquiring additional driver mutations in discrete evolutionary branches, the *TP53*-mutated cell population derived from that stem cell persisted > 50 years without evolving to cancer and inhabited a ~ 6.6 mm² PNE region in the 70-year-old subject. In other clones, driver mutations

were frequently acquired before 20 years of age (**Extended Fig. 10**). Importantly, when evolutionary patterns were compared between clones observed in high- (n=17) and low-risk (n=12) elderly individuals, substantial differences were observed; in high-risk subjects, evolutionary trees are characterized by a significantly taller tree height, more branches, a longer total length of branches, and a higher number of driver mutations (**Fig. 6b and Extended Fig. 10**), suggesting that lifestyle risks significantly accelerated clonal evolution. Compared with the mutations acquired in early life (<20 years), those in later life (>20 years) exhibited an increased number of Sig. D mutations, which was not observed in low-risk individuals (**Fig. 6c**), suggesting a contribution of lifestyle ESCC risk in adult life, most likely heavy drinking, to accumulation of mutations.

Discussion

Through an unbiased detection of somatic mutations in PNE from a large number of subjects, we demonstrated a comprehensive landscape of clonal expansion in PNE and further revealed ultrastructure of expanded clones with their evolutionary history. Remodelling of oesophageal epithelia by numerous driver-mutated clones is an inevitable consequence of normal aging (hence, age-related clonal expansion in oesophageal epithelia; ARCE). ARCE emerges multifocally in virtually all individuals as early as in early infancy, without any exposure to lifestyle ESCC risks, which however, is substantially accelerated by heavy alcohol drinking and smoking. Over years, evolving with their own phylogenetic structures, driver-mutated clones increase their number and size and in the extreme elderly, almost entire oesophageal epithelia are remodelled by thousands of sizable driver-mutated clones (~9,000–15,000/oesophagus).

Despite a large number of driver-mutated clones in every elderly individual, an estimated lifetime incidence of ESCC remains only ~1/45-1/228, which is in large part limited to heavy drinkers and smokers²⁶, suggesting a critical role of these lifestyle risks on ESCC development. Given the strong impact of lifestyle ESCC risk on the number and type of driver mutations positively selected in ARCE, of particular interest is a previous study showing a critical role of mutagens in tumorigenesis in NOTCH1-deficient skin, where treatment with 7,12-dimethylbenz[a] anthracene was essential for the accelerated tumour development in *Notch1*-deleted mouse skin²⁷. Importantly, in this model, mutagen-induced tumours developed even from residual *Notch1*-undeleted cells, suggesting non-cell autonomous effects of Notch1-deficient cells. This raises a possibility that the field effects could be explained by ARCE exposed to lifestyle ESCC risk, where expanded multiple *NOTCH1*-mutated clones provide a milieu for tumour development accelerated by heavy alcohol drinking and smoking, well known mutagenic agents.

Finally, the current observations might raise a question: what is the essential difference in clones in PNE and cancer? Our knowledge is still limited regarding the mechanism of clonal evolution in normal tissues and the way it differs from cancer evolution, which would be important issues to be addressed to understand cancer and to develop early detection, intervention, and even prevention of ESCC in high-risk patients.

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AUTHOR CONTRIBUTIONS

A.Y., H.Suzuki, T.Y., N.K., H.M., M.M., and S.Ogawa designed the study. S.Ohashi, Y.A., I.A., T.H., S.Miyamoto, S.T., Y.Sakai, Yoshitaka Sato, H.Seno, M.M., and Y.Nishikawa provided specimens. Y.T., S.Minanigushi, and H.H. performed histological analysis. A.Y., H.Suzuki, T.Y., N.K., Y.Shiozawa, Yusuke Sato, K.A., Y.T., S.K.K., Y.F., K.Y., K.K., Y.I., T.Hirano, and M.M.N. performed sample preparation. G.S. and K.M. provided sequencing data. M.S., A.Y., H.Suzuki, T.Y., N.K., Y.Shiozawa, and Y.Nannya performed mutation calling, validated the results, and analysed copy number alterations, mutational signature, and clonal dynamics. A.Y., H.Suzuki, T.Y., N.K., Y.Shiozawa, K.A., Y.T., Y.Nannya, Y.Shiraishi, K.C., H.T., M.N., J.B.B., and S.Miyano performed bioinformatics analysis. A.Y., H.Suzuki, T.Y., N.K., Y.Nannya, H.M., and S.Ogawa prepared the manuscript.

Competing financial interests

The authors declare no competing financial interests.

References

- 1 Greaves, M. & Maley, C. C. Clonal evolution in cancer. *Nature* **481**, 306-313, doi:10.1038/nature10762 (2012).
- 2 McGranahan, N. & Swanton, C. Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future. *Cell* **168**, 613-628, doi:10.1016/j.cell.2017.01.018 (2017).
- 3 Gerlinger, M. *et al.* Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med* **366**, 883-892, doi:10.1056/NEJMoa1113205 (2012).
- 4 Cooper, C. S. *et al.* Analysis of the genetic phylogeny of multifocal prostate cancer identifies multiple independent clonal expansions in neoplastic and morphologically normal prostate tissue. *Nat Genet* **47**, 367-372, doi:10.1038/ng.3221 (2015).
- 5 Jaiswal, S. *et al.* Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med* **371**, 2488-2498, doi:10.1056/NEJMoa1408617 (2014).
- 6 Genovese, G. *et al.* Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med* **371**, 2477-2487, doi:10.1056/NEJMoa1409405 (2014).
- 7 Martincorena, I. *et al.* Universal Patterns of Selection in Cancer and Somatic Tissues. *Cell* **171**, 1029-1041 e1021, doi:10.1016/j.cell.2017.09.042 (2017).
- 8 Martincorena, I. *et al.* Tumor evolution. High burden and pervasive positive selection of somatic mutations in normal human skin. *Science* **348**, 880-886, doi:10.1126/science.aaa6806 (2015).
- 9 Pickens, A. & Orringer, M. B. Geographical distribution and racial disparity in esophageal cancer. *Ann Thorac Surg* **76**, S1367-1369 (2003).
- 10 Enzinger, P. C. & Mayer, R. J. Esophageal cancer. *N Engl J Med* **349**, 2241-2252, doi:10.1056/NEJMra035010 (2003).
- 11 Matejicic, M., Gunter, M. J. & Ferrari, P. Alcohol metabolism and oesophageal cancer: a systematic review of the evidence. *Carcinogenesis* **38**, 859-872, doi:10.1093/carcin/bgx067 (2017).
- 12 Ishiguro, S. *et al.* Effect of alcohol consumption, cigarette smoking and flushing response on esophageal cancer risk: a population-based cohort study (JPHC study). *Cancer Lett* **275**, 240-246, doi:10.1016/j.canlet.2008.10.020 (2009).

377 13 Katada, C. *et al.* Alcohol Consumption and Multiple Dysplastic Lesions Increase
378 Risk of Squamous Cell Carcinoma in the Esophagus, Head, and Neck.
379 *Gastroenterology*, doi:10.1053/j.gastro.2016.07.040 (2016).

380 14 Tian, D. *et al.* Multifocal accumulation of p53 protein in esophageal carcinoma:
381 evidence for field cancerization. *Int J Cancer* **78**, 568-575 (1998).

382 15 Chai, H. & Brown, R. E. Field effect in cancer-an update. *Ann Clin Lab Sci* **39**,
383 331-337 (2009).

384 16 Sawada, G. *et al.* Genomic Landscape of Esophageal Squamous Cell Carcinoma
385 in a Japanese Population. *Gastroenterology*, doi:10.1053/j.gastro.2016.01.035
386 (2016).

387 17 Cancer Genome Atlas Research, N. *et al.* Integrated genomic characterization
388 of oesophageal carcinoma. *Nature* **541**, 169-175, doi:10.1038/nature20805
389 (2017).

390 18 Chang, J. *et al.* Genomic analysis of oesophageal squamous-cell carcinoma
391 identifies alcohol drinking-related mutation signature and genomic alterations.
392 *Nat Commun* **8**, 15290, doi:10.1038/ncomms15290 (2017).

393 19 Gao, Y. B. *et al.* Genetic landscape of esophageal squamous cell carcinoma. *Nat*
394 *Genet* **46**, 1097-1102, doi:10.1038/ng.3076 (2014).

395 20 Qin, H. D. *et al.* Genomic Characterization of Esophageal Squamous Cell
396 Carcinoma Reveals Critical Genes Underlying Tumorigenesis and Poor Prognosis.
397 *Am J Hum Genet* **98**, 709-727, doi:10.1016/j.ajhg.2016.02.021 (2016).

398 21 Alexandrov, L. B. *et al.* Signatures of mutational processes in human cancer.
399 *Nature* **500**, 415-421, doi:10.1038/nature12477 (2013).

400 22 Totoki, Y. *et al.* Trans-ancestry mutational landscape of hepatocellular
401 carcinoma genomes. *Nat Genet* **46**, 1267-1273, doi:10.1038/ng.3126 (2014).

402 23 Blokzijl, F. *et al.* Tissue-specific mutation accumulation in human adult stem
403 cells during life. *Nature*, doi:10.1038/nature19768 (2016).

404 24 Lawrence, M. S. *et al.* Mutational heterogeneity in cancer and the search for
405 new cancer-associated genes. *Nature* **499**, 214-218, doi:10.1038/nature12213
406 (2013).

407 25 Bulavin, D. V. *et al.* Inactivation of the Wip1 phosphatase inhibits mammary
408 tumorigenesis through p38 MAPK-mediated activation of the p16(Ink4a)-
409 p19(Arf) pathway. *Nat Genet* **36**, 343-350, doi:10.1038/ng1317 (2004).

410 26 Hori, M. *et al.* Cancer incidence and incidence rates in Japan in 2009: a study
411 of 32 population-based cancer registries for the Monitoring of Cancer Incidence
412 in Japan (MCIJ) project. *Jpn J Clin Oncol* **45**, 884-891, doi:10.1093/jjco/hyv088
413 (2015).

414 27 Demehri, S., Turkoz, A. & Kopan, R. Epidermal Notch1 loss promotes skin
415 tumorigenesis by impacting the stromal microenvironment. *Cancer Cell* **16**, 55-
416 66, doi:10.1016/j.ccr.2009.05.016 (2009).

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Figure Legends

Figure 1. WES of PNE, cancer, and dysplasia samples

a, b. Number (**a**) and maxMCF (**b**) of mutations detected by WES in low- (blue) and high-risk (red) samples are plotted against subject's age according to sampling size. Numbers of low- and high-risk samples in 0.2 mm², 0.8 mm² and 4 mm² sampling size are 34 and 19, 19 and 12, and 40 and 33 (**a**) and 34 and 19, 18 and 12, and 37 and 31 (**b**), respectively. Regression lines are shown for low-risk samples with R². *P*-values show significant difference between risk (+) and (–) samples (one-sided Mann-Whitney U test). **c.** Four predominant mutational signatures (Sigs. A-D) revealed by PMsignature analysis of 54,121 SNVs detected in 688 samples, including 157 PNE, 12 dysplasia, and 519 cancer samples. Information for strand bias is shown on the right. **d.** The mean number and composition of mutations assigned to Sigs. A-D in low- and high-risk PNE, dysplasia, and cancer samples having ≥ 10 mutations.

Figure 2. WGS of single cell-derived colonies

a. Total number and signature composition of SNVs detected by WGS in single cell-derived colonies from PNE in individuals of indicated age and lifestyle ESCC risk. **b.** Strand bias for indicated base substitutions. **c, d.** Number of mutations across the entire genome (**c**) or exome (**d**) is plotted against subject's age. **e.** Correlation between age and the number of mutations assigned to Sigs. A-D. In **c-e**, regression lines are indicated for low-risk samples with R², and *P*-values are for significant difference between colonies from high- (n=3) and low-risk (n=10) individuals (two-sided Mann-Whitney U test).

Figure 3. Driver mutations in PNE and ESCC samples

a. Frequencies of major driver mutations among PNE (n=157) and cancer (n=519) samples. Orange bars indicate drivers detected in each sample set (**Online Methods**). Drivers showing significantly different mutation frequencies between PNE and cancer are indicated by asterisks (two-sided Fisher's exact test with B-H adjustment; *q*<0.05). **b.** Distribution of *NOTCH1*, *TP53*, *PPM1D*, and *ZFP36L2* mutations in PNE (top) and cancer (bottom) samples. **c.** Forest plots showing enrichment of driver mutations between PNE samples from low- (n=93) and high-risk (n=64) individuals with 95%CI. Significant enrichment is shown in red (two-sided Fisher's exact test with B-H adjustment for multiple testing; *q*<0.05). Asterisks indicate the significant

enrichment adjusted for age (two-sided ANOVA). Plots are shown for 21 genes mutated in both low- and high-risk samples. **d.** Fractions of PNE samples from individuals (≥ 50 y.o.) having indicated numbers of driver mutations according to histology, sample size, and lifestyle ESCC risks. Plots are also provided for dysplasia and cancer samples (4 mm²).

Figure 4. Fine architecture of clones in high-density sampling

a. For each mutation found in at least two samples from each biopsy specimen, average MCF among the samples sharing the mutation (average MCF) and the size of the smallest circle that encompasses all mutation (+) samples are plotted. **b,c.** Spatial distribution of clones is depicted for representative biopsies from a 23-year-old low-risk male (UPN95) (**b**) and an 81-year-old (UPN59) low-risk male (**c**). Samples sharing different sets of mutations are discriminated by colour, where maxMCF in the corresponding set of shared mutations is indicated by arch length (top panel in **b** and **c**) and is summarized for one subject (bottom panel in **b**). MCFs of driver mutations in each sample are also depicted in separate sampling maps using the same colours that correspond to the parental clones (**see also Extended Fig. 8**). Calibration is provided for each specimen.

Figure 5. Expansion of driver-mutated clones

a. Density of driver-mutations expanded in two or more samples and their average size are plotted against subject's age for each biopsy specimen. Regression lines for low-risk samples is indicated with corresponding correlation coefficients and R^2 (blue letters). *P*-values are for significance between low- and high-risk samples (one-sided Mann-Whitney U test). Number of independent *TP53* (T) and *NOTCH1* (N) mutations in each biopsy specimen is also depicted. **b.** Fraction of total sampling area occupied by the indicated number of driver mutations is plotted for each biopsy specimen. **c.** Mutation density for each mutation (top) and fraction of total sampling area occupied by indicated mutations (bottom) are plotted for biopsy specimen from high- and low-risk individuals. Subject's age, sex, and exposure to lifestyle ESCC risks are indicated in the bottom panels.

Figure 6. Clonal evolution in PNE

a. Evolution of two representative clones (out of 29 clones from 5 biopsies) found in PNE samples from high-risk 81-year-old (left panels) and 70-year-old males (right panels). Phylogenetic trees of clones (middle panels) are projected onto the positions of samples as indicated by real images of biopsy specimens (upper panels). Estimated age of branching on the basis of the mutation rate in PNE cells (**Fig. 2**) is also shown. Imputed clonal structures are depicted at the bottom, where estimated frequencies of imputed mutational clusters (vertical axis) in each sample are shown as the distribution of posterior probabilities (width of the violin plots) calculated from the Pyclone model. Colours correspond to those in the associated phylogenetic trees. **b.** Boxplots of the number of evolutionary branches, the maximum and total lengths of branches, and the total number of driver mutations between high- (n=17) and low-risk (n=12) clones, in which median and first and third quartiles are indicated with whiskers extending to the furthest value within 1.5 of IQR. *P*-values for significant differences are from two-sided Mann-Whitney U test. **c.** Mutational signatures of mutations in early (< 20 y.o.) vs. late (>20 y.o.) branches are shown for clones in one low-risk and three high-risk individuals.

Methods

Subjects and materials

We enrolled a total of 139 patients who underwent surgery and/or therapeutic or diagnostic endoscopy for upper gastrointestinal symptoms at Kyoto University Hospital and other participating institutes. Among these, 95 had ESCC (n = 93) or high-grade oesophageal dysplasia (n = 2) and the remaining 44 were turned out to have no oesophageal lesions and thought to be healthy individuals. Informed consent was obtained from all participants. Characteristics of these patients and healthy individuals are summarized in **Supplementary Table 1**. History of heavy alcohol drinking (≥ 396 grams alcohol/week) or tobacco smoking (≥ 30 pack-years) was reported in 95 individuals, who were considered to have positive lifestyle risks for ESCC^{10,28} (high-risk individuals). None of the patients included had received prior chemoradiotherapy. This study comprises following 4 sequencing experiments (Exp. 1-4) (**Extended Fig. 1 and Supplementary Tables 1 and 2**):

Exp. 1) Whole exome sequencing (WES) of solitary physiologically normal oesophageal epithelia (PNE)/cancer samples, including those obtained by multi-regional sampling (189 ESCC/dysplasia/PNE samples from 62 subjects and 27 ESCC/PNE samples from a surgical specimen), followed by targeted deep re-sequencing of 24 driver genes for PNE samples,

Exp. 2) Whole genome sequencing (WGS) of single cell-derived colonies (13 samples from 7 subjects),

Exp. 3) Deep sequencing of 24 driver genes in cryo-archived samples of matched PNE/cancer (136 samples from 68 patients),

Exp. 4) Whole exome or targeted panel sequencing of densely collected samples (16 samples from 14 subjects),

In Exp. 1-4, samples were obtained from overlapped subjects across the different experiments except for Exp. 1 and Exp. 3. This study was approved by the ethical committee at Kyoto University.

Biomaterials including oesophageal tissues and peripheral blood, were newly collected from 71 individuals using endoscopic biopsy or stereoscope-guided sampling from a surgical specimen, according to the procedures approved by the Internal Review Board at Kyoto University (G0645). For the remaining 68 patients, a cryo-preserved

oesophageal biopsy with paired matched blood were provided from the Kyoto University Hospital Cancer Biobank (No.1056). Endoscopic biopsy from multiple sites were performed in 17 individuals with a median of 5 biopsies per subject (range: 2–8) for Exp. 1), 2), and 4). All subjects and samples were anonymized with unique IDs before analysis. All the specimens obtained from cancer and dysplasia patients excluding cryo-preserved samples were reviewed independently by two expert pathologists (Y.T., and S.M.) at the Department of Diagnostic Pathology, Kyoto University, blinded for clinical information, to confirm histology based on the WHO Classification of Tumours of the Digestive System (4th Edition). Accordingly, all the samples were categorized into either of the four pathological groups; PNE, low- and high-grade dysplasia, and cancer.

For sequencing analysis of oesophageal tissues, we prepared a total of 559 samples from the newly obtained biopsy specimens under dissection microscope. Among these, 27 (25 PNE and 2 cancer samples) were from the surgically resected oesophagus from a 58-year-old high-risk ESCC patient with a long history of heavy smoking (UPN32). The remaining 532 samples were prepared from 166 endoscopy biopsies, using razor (for 4 mm² samples; n = 118) or disposable biopsy punches BP-10F (for 0.8 mm² samples; n = 31) or BP-A05F (for 0.2 mm² samples, n = 383) (Kai medical) and included 157 PNE, 12 dysplasia, and 20 cancer samples for Exp. 1, and 13 and 341 PNE samples for Exp. 2 and 4, respectively (**Extended Fig. 1**). To enhance the sensitivity of mutation detection, submucosal layers were removed from each biopsy specimen before DNA extraction (**Extended Fig. 2e**), except for the samples from surgically obtained specimens and archived frozen specimens. Peripheral blood samples were collected from all cases and used as germline controls. In particular, 16 biopsy specimens from 14 individuals were subjected to high-density sampling, through which a median of 22 (range: 8–34) samples were collected from a single biopsy with a uniform sampling size of 0.2 mm² (**Supplementary Table 3**).

These samples, together with archived samples of paired PNE/cancer and matched peripheral blood from 68 ESCC patients (n = 204), were subjected to extraction of genomic DNA with the DNeasy Tissue Kit (Qiagen) or QIAamp DNA Micro Kit (Qiagen) according to the manufacture's protocols. Sample information are summarized in **Supplementary Table 2**.

External data sets

WES data (bam files) of paired tumour/germline control samples from ESCC (n = 90) and other cancer (n = 8,808) patients were downloaded from the TCGA Data Portal (<https://tcga-data.nci.nih.gov/docs/publications/tcga/>). WES data of a Japanese cohort of 142 ESCC patients (Kyushu cohort) have previously been published¹⁶. The clinical data of these patients were summarized in **Supplementary Table 1**. These bam files from the TCGA and the Kyushu cohorts were converted to fastq format using biobambam (<https://github.com/gt1/biobambam>) and processed with the same pipeline for mutation calling as applied to the Kyoto University cohort (see below). Mutation lists of 272 ESCC samples were available from the publications of an additional three ESCC WES studies¹⁸⁻²⁰.

Whole-exome sequencing

WES libraries were generated using SureSelect Human All Exon v5 (Agilent Technologies), followed by sequencing of enriched exon fragments on Hiseq 2500 or NovaSeq 6000 (Illumina) with 100–150 bp paired-end mode as previously described²⁹. Depending on the purpose of experiments, the target depth was 150× (Exp. 1 and 4), for which the actual coverage was 185× (54x-425x) and 148× (47x–511x) on average, respectively. The mean coverage in blood samples from 78 individuals was 144× (89x–233x). Mutation calling was performed using Genomon2 pipeline (URL: <https://genomon.readthedocs.io/ja/latest/>) (**Supplementary Method 1**), as previously described³⁰. Briefly, sequencing reads were aligned to the human genome reference (hg19) using Burrows-Wheeler Aligner, version 0.7.10 with default parameter settings. PCR duplicates were eliminated using picard-tools version 1.39 (<http://picard.sourceforge.net/>). Somatic mutations were detected by eliminating polymorphisms and sequencing errors. To achieve this, Genomon2 first discards any of low-quality, unreliable reads and variants, that are defined by the following criteria: (i) mapping quality < 30, (ii) base call quality < 15, (iii) any reads having ≥ 5 single nucleotide variants (SNVs) or indels. After further excluding those variants that are not supported by a sufficient number of reads (total reads ≥ 10 and variant reads ≥ 3), variant allele frequencies (VAFs) ≥ 0.05 (for tissue sample) and ≤ 0.02 (for normal control), and strand ratio ≠ 0 or 1, the remaining variants are interrogated for the evidence that they are observed at significantly higher VAFs than expected for errors

($P \leq 10^{-5}$), where the significance is evaluated by EBCall algorithm³¹, on the basis of an empirical VAF distribution as determined using WES data of non-paired peripheral blood samples from the same sequencing run ($n = 15-20$). Putative germline variants are also excluded by comparing VAFs with matched control using the Fisher's test ($\leq 10^{-2}$)³², which also eliminates remaining additional sequencing errors. All variants within repetitive sequences were excluded to obtain higher true positive rate at the expense of sensitivity. In total, 8,056 candidate somatic mutations were called by Genomon2 pipeline.

Some modifications were made to the original pipeline. Mutations clustered within a short bp length (150 bp) and other mutations found across multiple samples were subjected to visual inspection on the Integrative Genomics Viewer (IGV)³³, to further eliminate sequencing errors. Finally, we rescued mutations in driver genes, which were excluded by the stringent filters in the original mutation calling, applying less stringent filters with EBCall P -value $\leq 10^{-3}$ and Fisher's P -value $\leq 10^{-0.5}$.

For samples from high-density collection (Exp. 4), each of the mutations detected in a specimen was exhaustively interrogated in all the remaining samples; if a variant detected in one or more samples by EBCall was also present (VAF ≥ 0.05) in another sample within the biopsy specimen, the variant was considered to be true positive, because it has a high prior probability for true positivity there.

Validation of detected mutations

Validation of mutations detected by WES and WGS was performed using PCR-based deep ($>500\times$) sequencing as previously described²⁹, in which 622 SNVs and indels from WES and 94 from WGS were selected and evaluated. A mutation was considered to be validated, when (1) the sequencing depth was ≥ 500 in both test and germline control samples, (2) the VAF in the test sample was 5 times larger than that in the corresponding germline control sample, and (3) the VAF in the test sample should be large enough (≥ 0.01) to be captured by the lower threshold in WES (0.05)³². The overall validation rate was 95.7 % for WES (544 / 569 SNVs and 51 / 53 indels) and 94.7 % for WGS (86 / 89 SNVs and 3 / 5 indels). The results of validation sequencing were summarized in **Supplementary Table 4**.

Detection of significantly mutated (driver) genes

Significantly mutated genes, or driver genes, were detected by globally evaluating the number of nonsynonymous mutations with correction for gene size and local mutation rate using MutSigCV²⁴ with default setting. Driver genes were also investigated relying on dN/dS, which was calculated for all recurrently mutated genes, as well for all mutations detected by WES, using dNdScv⁷ (<https://github.com/im3sanger/dndscv>). After independently applying each of these two packages to the mutations in 519 ESCC and 157 PNE samples, those 24 genes that were significantly mutated by either MutSigCV or dNdScv analysis ($q < 0.05$) and found in 2% of PNE or ESCC cohorts were considered as drivers (**Supplementary Tables 5, 6 and 7**).

Deep targeted sequencing of driver genes (Exp. 1, 3, 4)

Targeted deep sequencing was performed according to the manufacture's protocols, using a custom bait library (xGen Predesigned Gene Capture Pool; IDT) that were designed to capture all 24 driver genes (**Supplementary Table 7**). Corresponding germline DNA from peripheral blood was also analysed unless it had been analysed by WES. After hybridization capture, the enriched DNA fragments were analysed on HiSeq 2500 or NextSeq 500 as previously described²⁹. Except for paired cancer/PNE samples, all DNA samples were whole genome-amplified before sequencing using REPLI-g Mini Kit (Qiagen). Mutation calling in targeted-capture was almost the same as that in WES, except that EBCall P -value $< 10^{-4}$, and Fisher's P -value $< 10^{-1}$ were used. Details of samples analysed with targeted deep sequencing are summarized in **Supplementary Tables 1, 2 and 8**.

Analysis of copy number abnormalities (CNAs)

CNAs were investigated using single nucleotide polymorphism (SNP) array and/or sequencing data (**Supplementary Table 1 and 2**). SNP array analysis was performed for paired cancer/non-cancer tissue samples using GeneChip Human Mapping 250K NspI (Affymetrix) according to the manufacture's instructions. All the SNP array data were uniformly analysed for CNAs using CNAG^{34,35}. For other samples which were analysed by whole-exome or targeted-capture sequencing with CN-bait (**Supplementary Table 7**), which included 'copy-number baits' designed for 1,760 SNP sites, CNAs were evaluated from sequencing data using our inhouse pipeline "CNACS"³⁶. Significant focal CNAs ($q < 0.25$) were detected using GISTIC 2.0³⁷ with a default setting based on the copy number data obtained above (**Supplementary Table 9**).

Estimation of mutant cell fractions

Precise estimation of the cell fraction having a somatic mutation, mutant cell fraction (MCF), might be complicated when the mutation involves a gene located on the chromosomal segment showing an abnormal copy number or an allelic imbalance³⁸. Fortunately, most PNE samples had no copy number abnormality or allelic imbalance existed at the mutated locus. Thus, for most mutations in PNE samples (5785/6128), MCF is simply estimated from the observed variant allele frequency (VAF) as follows:

$$\text{MCF} = 2 \times \text{VAF} \text{ (for autosome and X chromosome in female), or}$$

$$\text{MCF} = \text{VAF} \text{ (for X chromosome in male)}$$

By contrast, when the mutated locus did show abnormal copy number or allelic imbalance, estimation of MCF depends on several factors, which may not be inferred in a deterministic way, particularly in the case where a sample contains multiple clones with different mutant allele compositions. However, the vast majority of CNAs in PNE samples were 9q UPD and 17p UPD/deletion targeting mutated *NOTCH1* (n = 155) and *TP53* (n = 23), respectively, where mutations are thought to precede respective CNAs in most cases. Thus, for *TP53* and *NOTCH1* mutations in 17p LOH- and 9q UPD-positive samples, which accounted for 2.9% (178/6128) of all detected mutations,

$$\text{MCF} = 2 \times \text{VAF} - \% \text{UPD} / 100 \text{ (for UPD)}$$

$$\text{MCF} = \text{VAF} \times (2 - \% \text{Del} / 100) \text{ (for deletion)}$$

where %UPD and %Del denote estimated fraction (%) of the respective abnormality. Other mutations associated with CNAs accounted only for 2.7% (n = 165) of mutations in PNE, of which only 5 were mutations affecting drivers, we could provisionally estimate MCFs according to the formula for mutations associated with normal copy number, without substantially affecting the overall conclusions. Every analysis of maximum values of MCF (max MCF) presented in Figures was performed using the data only from samples with mutations. Samples without mutations were excluded from the subjects of max MCF analysis.

Analysis of mutational signature

Mutational signatures of 54,121 SNVs in 157 PNE, 12 dysplasia, and 519 cancer samples were extracted using R package, pmsignature³⁹. For multiple samples which were collected from the same cancer specimen, the sample which was collected at or nearest to the centre of specimen was subjected to the analysis. For comparison with the COSMIC signatures, mutations were allocated to 30 COSMIC mutational patterns using R package MutationalPatterns⁴⁰. The frequency of each signature was compared between PNE, dysplasia, and cancer samples. The effects of subject's age and lifestyle ESCC risks on the number of mutations assigned to each signature were evaluated as described in Statistical analysis. Genotyping of the two SNPs in *ALDH2* (rs671) and *ADH1B* (rs1229984) was determined for 8,898 TCGA samples and 162 Japanese ESCC samples based on the WES data of corresponding normal controls, in which samples were classified as homozygote if VAFs for either A or B allele exceeded 0.8 or otherwise, as heterozygote. VAFs of each polymorphic allele were calculated by performing local realignment around SNPs using GenomonMutationFilter realignment (<https://github.com/Genomon-Project/GenomonMutationFilter>) with the default setting. Samples with less than 20 reads were excluded from the analysis. The frequency and the number of mutations assigned to COSMIC signature 16 was compared in 162 Japanese ESCC samples between *ALDH2/ADH1B* genotypes with or without alcohol drinking using Mann-Whitney U test. The effect of alcohol drinking and smoking on the number of Sig. C mutations was also compared in the same manner. For TCGA pan-cancer cohort, we compared the number of mutations allocated to COSMIC signature 16 between *ALDH2* genotypes in each cancer type.

Whole genome sequencing of single colony-derived sample

To measure the mutation rate at the single cell level and its age-dependence, we isolated 13 single PNE cells from 5 low-risk healthy individuals and 2 high-risk ESCC patients by limiting dilution after 3-5 days of primary culture from biopsy specimens. Isolated single cells were further cultured with KSFM supplemented with 50ng/mL BPE and 1ng/mL EGF for 2-3 weeks, followed by DNA extraction, in which the median recovery was 92 ng (range: 40-399 ng) per single cell culture. Library preparation for whole genome sequencing was performed using KAPA Hyper Prep Kit, followed by sequencing with 63× depth using NovaSeq 6000 with matched blood DNA (35×). Genomon2 pipeline was used for mutation calling with the same parameters as used

for WES, except that EBCall, Fisher *P*-values, and VAFs were $\leq 10^{-6}$, $\leq 10^{-1}$, and ≥ 0.25 , respectively. Detected mutations were allocated to Sigs. A-D, which were identified in PNE/ESCC samples, using MutationalPatterns⁴⁰.

Detection of clones in high-density sampling

For each biopsy specimen, discrete sets of samples were constructed so that all the samples in each set shared at least one mutation that were not found in the remaining samples. For each set of samples, clonal composition was interrogated based on the Beta Binomial emission model implemented in PyClone version 0.13.0⁴¹, through which a set of clones having a discrete set of mutations were imputed together with their estimated clone size. These clones are then used to reconstruct a hierarchical tree. In general, any two mutations (mut_a and mut_b) were considered to belong to two independent clones, if they were spatially separated, i.e., if there existed two samples having different mutations, i.e., mut_a alone and mut_b alone, respectively. By contrast, if the sum of MCFs of two mutations ($d^A \geq d^B$ for mutations A and B) in a given sample exceeded 1, the sample was considered to contain two clones harbouring either both mutations and mutation A alone, in fractions of d^B and $d^A - d^B$, respectively (pigeonhole principle). In addition, if a set of mutations were co-segregated into ≥ 3 spatially separated samples, the presence of a clone harbouring all those mutations was indicated. When clones did not satisfy any of the above criteria, clonal architecture could not be determined and d^B clones were excluded from the relevant analyses.

After reconstruction of hierarchical trees, number of branches, the maximum and total lengths of branches, and the total number of driver mutations were calculated for each clone in high- and low-risk elderly subjects (**Supplementary Table 10**). Mutations belonging to each branch of clones were divided into early (acquired at <20 y.o.) and later mutations (>20 y.o.) according to the mutation rate in exon region (0.531 and 0.8098 mutation per year for low risk and high-risk cases, respectively) that was estimated from WGS of single cell-derived colonies. Mutations belonging to the branches which had 10 or less mutations together with their ancestor branches were treated as those acquired in early life. Mutations belonging to the branches whose ancestor branches had 11 or more mutations were deemed as late events. Early and late mutations were allocated to Sigs. A-D (see above), using MutationalPatterns

independently.

Criteria for bi-allelic mutations

Bi-allelic mutations were thought to be present, if

- (1) Sum of MCFs of multiple mutations affecting a same gene exceeded 1.0, or
- (2) Locus of the mutated gene showed LOH.

Measurement of the areas of mutant clones by high-density sampling

In high-density micrometre-scale (0.2 mm^2) sampling, many mutations were observed in multiple samples particularly in elderly and high-risk cases. The expansion of each mutant clone in high-density sampling was evaluated in terms of 1) the sum of MCFs $\times$ sampling area (0.2 mm^2) for each of the affected samples and 2) the size of the minimum circle that encompassed all the mutation(+) samples ('minimum circle'). The size of the minimum circle was calculated for all mutations shared by $2 \geq$ samples, where each sample was considered to be a 0.2 mm^2 circle located at the coordinate recorded during micro-scale biopsy (**Fig. 4a**). In analysis of density of clones with driver mutations in high-density sampling, the minimum circle that encompassed all the samples was used as the area of each specimen and the clone size of mutations observed only in 1 sample were treated as a 0.2 mm^2 circle (**Fig. 5a**).

Estimation of mean cell density in PNE

The amount of DNA in a human somatic cell can be calculated relying on the mean recovery of DNA from punch biopsy. To minimize the effect of lower DNA recovery anticipated for small samples, the mean recovery from 4 mm^2 samples (1,223 ng/sample) (**Extended Fig. 2g**) was used for the calculation, assuming a typical recovery of $\sim 90\%$. Assuming $\sim 6.6 \text{ pg}$ DNA per single diploid cell, we estimated the mean cell density of $\sim 51,500 \text{ cell/mm}^2$ or $10,300 \text{ cells}/0.2 \text{ mm}^2$.

Statistical analysis

Statistical analyses were performed using R version 3.5.1. All *P*-values were calculated by two-sided analysis unless otherwise specified. Fisher's exact test or Mann-Whitney U test were used for group comparisons. Confidence intervals of frequency of positive samples were estimated using binomial test. Multiple testing was corrected based on Benjamini-Hochberg *q* values. Independent effects of

lifestyle ESCC risk on the enrichment of mutations in high-risk individuals were tested in driver genes which were mutated in 5% or more of the PNE samples by evaluating the improvement in the logistic regression model incorporating both age and lifestyle ESCC risk vs. age alone, using ANOVA. Linearity of the number of mutations and MCFs with age was evaluated based on the Pearson's correlation coefficient in a linear regression model assuming zero intercept for risk(-) samples, but not for risk(+) samples, because the exposure to ESCC risks begins usually after the adolescent. Significance of correlation of age to these variables in risk(-) samples were tested using F test. Significance of the effect of lifestyle ESCC risk on the number of mutations, MCFs, and frequencies of samples with driver mutation was tested using the one-side Welch's t-test under the null hypothesis: residuals in the regression model for risk-positive samples follows the same distribution with those for risk-negative samples, where 0 residual was treated as a negative value. Observations in the different sample size were combined using a random effects model meta-analysis with R package "metaphor"⁴², in which the mean difference of residuals between risk(+) and risk(-) samples were standardized across different sample sizes without assuming equal variance and tested for significance. Correlation coefficients for age in the different sample size among risk(-) samples were combined with Fisher's r-to-z transformation. Correlation coefficients for age in the different sample size among risk(-) samples were combined with Fisher's r-to-z transformation.

Code Availability

CNACS is a UNIX-based program for sequencing-based copy number analysis, which is available from our website (http://plaza.umin.ac.jp/kyoto_tumorpatho/CNACS.html). A custom R script for a random effects model meta-analysis is available in **Supplementary Method 2**.

Data Availability

All the WES, WGS, and SNP array data have been deposited in the European Genome-Phenome Archive (<http://www.ebi.ac.uk/ega/>) under accession numbers EGAS00001003008, EGAS00001003281, and EGAS00001003331, respectively. Data for Figs. 1ab, 2, 3c, 4a, 5, 6c is available as source data. All data are available from the corresponding author on reasonable request.

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815 **References**

- 816 28 Katada, C. *et al.* Alcohol Consumption and Multiple Dysplastic Lesions Increase
817 Risk of Squamous Cell Carcinoma in the Esophagus, Head, and Neck.
818 *Gastroenterology* **151**, 860-869 e867, doi:10.1053/j.gastro.2016.07.040 (2016).
- 819 29 Yoshida, K. *et al.* Frequent pathway mutations of splicing machinery in
820 myelodysplasia. *Nature* **478**, 64-69, doi:10.1038/nature10496 (2011).
- 821 30 Kataoka, K. *et al.* Aberrant PD-L1 expression through 3'-UTR disruption in
822 multiple cancers. *Nature*, doi:10.1038/nature18294 (2016).
- 823 31 Shiraishi, Y. *et al.* An empirical Bayesian framework for somatic mutation
824 detection from cancer genome sequencing data. *Nucleic Acids Res* **41**, e89,
825 doi:10.1093/nar/gkt126 (2013).
- 826 32 Suzuki, H. *et al.* Mutational landscape and clonal architecture in grade II and
827 III gliomas. *Nat Genet* **47**, 458-468, doi:10.1038/ng.3273 (2015).
- 828 33 Thorvaldsdottir, H., Robinson, J. T. & Mesirov, J. P. Integrative Genomics Viewer
829 (IGV): high-performance genomics data visualization and exploration. *Brief*
830 *Bioinform* **14**, 178-192, doi:10.1093/bib/bbs017 (2013).
- 831 34 Nannya, Y. *et al.* A robust algorithm for copy number detection using high-
832 density oligonucleotide single nucleotide polymorphism genotyping arrays.
833 *Cancer Res* **65**, 6071-6079, doi:10.1158/0008-5472.CAN-05-0465 (2005).
- 834 35 Yamamoto, G. *et al.* Highly sensitive method for genomewide detection of
835 allelic composition in nonpaired, primary tumor specimens by use of affymetrix
836 single-nucleotide-polymorphism genotyping microarrays. *Am J Hum Genet* **81**,
837 114-126, doi:10.1086/518809 (2007).
- 838 36 Yoshizato, T. *et al.* Genetic abnormalities in myelodysplasia and secondary
839 acute myeloid leukemia: impact on outcome of stem cell transplantation. *Blood*
840 **129**, 2347-2358, doi:10.1182/blood-2016-12-754796 (2017).
- 841 37 Mermel, C. H. *et al.* GISTIC2.0 facilitates sensitive and confident localization
842 of the targets of focal somatic copy-number alteration in human cancers.
843 *Genome Biol* **12**, R41, doi:10.1186/gb-2011-12-4-r41 (2011).
- 844 38 Carter, S. L. *et al.* Absolute quantification of somatic DNA alterations in human
845 cancer. *Nat Biotechnol* **30**, 413-421, doi:10.1038/nbt.2203 (2012).
- 846 39 Shiraishi, Y., Tremmel, G., Miyano, S. & Stephens, M. A Simple Model-Based

847 Approach to Inferring and Visualizing Cancer Mutation Signatures. *PLoS Genet*
848 **11**, e1005657, doi:10.1371/journal.pgen.1005657 (2015).
849 40 Blokzijl, F., Janssen, R., Van Boxtel, R. & Cuppen, E. MutationalPatterns: an
850 integrative R package for studying patterns in base substitution catalogues.
851 *bioRxiv*, doi:10.1101/071761 (2016).
852 41 Roth, A. *et al.* PyClone: statistical inference of clonal population structure in
853 cancer. *Nat Methods* **11**, 396-398, doi:10.1038/nmeth.2883 (2014).
854 42 Viechtbauer, W. Conducting Meta-Analyses in R with the metafor Package. *J*
855 *Stat Softw* **36**, 1-48 (2010).
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Legends to Extended Figures

Extended Figure 1. Study design

Summary of the subjects, samples, methods of sample collections, lifestyle ESCC risks, and histology, as well as sequencing platforms used for analysis of **(a)** solitary samples, **(b)** single cell-derived colonies, and **(c)** densely collected samples.

* For one case (UPN53), only cancer and dysplasia samples were collected.

Samples from § UPN36, UPN37, UPN39, ¶ UPN49, ‡ UPN88, ⊥ UPN56, UPN61, Φ UPN58, †† UPN55, UPN59, UPN60, UPN65 were analysed in indicated multiple experiments (see panels **a**, **b**, and **c**).

† Eleven out of 53 PNE samples were analysed in both solitary and high-density sampling (see panels **a** and **c**).

Extended Figure 2. WES of multi-regional sampling from PNE and cancer in a surgical specimen

a. Positions of 25 PNE and 2 cancer samples from a surgically resected oesophagus in a 58-year-old high-risk ESCC patient (UPN32) are shown by green and red circles, respectively. Calibration is shown on the right. **b.** Mutated cell fractions (MCFs) are compared between samples from PNE (green) (n=25) and cancer (red) (n=2) samples. **c, d.** MCF is plotted for all shared (orange) and private (grey) mutations for low- **(c)** and high-density **(d)** sampling. Driver mutations are indicated by arrowheads in indicated colours. Position of each sample is shown on the left. Calibration for the number of mutations depicted in the horizontal axis is separately provided for each sampling. EGJ: esophagogastric junction. **e.** For preparation of PNE samples, after submucosal layers were removed from endoscopic biopsy specimens, 0.2 mm², 0.8 mm², or 4.0 mm² samples were collected using punch biopsy of 0.5 mm or 1.0 mm in diameter, or 2 × 2 mm rectangular excision, respectively, followed by DNA extraction. **f.** Real scale pictures of punch biopsy devices for Φ 0.5 mm and Φ 1.0 mm sampling. **g.** Histogram of DNA recovered from 0.2 mm², 0.8 mm², or 4.0 mm² samples. Boxplots represent the median, first quartile and third quartile with whiskers extending to the furthest value within 1.5×IQR.

Extended Figure 3. Representative results of WES for multiregional sampling

a. Summary of somatic mutations found in 157 PNE, 12 dysplasia, and 20 cancer samples from individuals with (pink) or without (green) lifestyle ESCC risks. Mutated

cell fractions (MCFs) are plotted for all mutations (upper panel). Driver mutations are shown by coloured triangles as indicated. Information about histology, sample size is shown on the top lanes. Subject's age and total number of mutations are shown in the bottom panels. Status of ESCC, copy number alterations (CNAs), and mutations are also shown (middle panels). **b-j.** MCFs of somatic mutations detected in WES of multiple samples from PNE as well as dysplastic and cancer tissues are depicted separately for shared (orange) and non-shared mutations (grey) in representative cases from 16 independent individuals from whom multi-regional sampling was performed. Mutations in *TP53*, *NOTCH1*, *PPM1D*, and other drivers are indicated by arrowheads in different colours. Positions of tumours and biopsy samples are shown on the left in each panel. Distance from the maxillary dental arch is indicated. Mutations are shared only between cancer samples, except in two cases (**i** and **j**), in which small numbers of mutations (3-16) are shared between mutually positioned samples as indicated. EGJ: esophagogastric junction.

Extended Figure 4. Signatures of mutations in PNE, ESCC, and dysplasia samples

a. Number (middle) and relative frequency (bottom) of mutations allocated to Sigs. A-D in the individual samples. Information about pathology, lifestyle ESCC risks, cohort, and subject's age is indicated (top). **b.** Number of mutations allocated to indicated mutational signatures is plotted against subject's age for low- (blue) and high- (red) risk PNE samples. Numbers of low- and high-risk samples in 0.2 mm², 0.8 mm², and 4 mm² sampling are 34 and 19, 19 and 12, and 40 and 33, respectively. Regression lines for low-risk samples are indicated with R². P-values for significance between risk (+) and (-) are also provided (one-sided Mann-Whitney U test). **c.** Mean±95%CI of standardized residuals of the number of mutations allocated to each signature in high-risk samples against the linear regression model in low-risk samples is plotted for 0.2 mm², 0.8 mm², and 4.0 mm² samples and for combined samples in RE model. P-value in the RE model is indicated, together with the weight from each sample size during the model fitting (two-sided Wald test) (Online Methods). **d.** Mean (± SD) number of mutations allocated to Sig. 16 in the current cohort of ESCC (n=90) (left) and those of hepatic (LIHC) (n=361) (middle) and stomach (STAD) (n=239) (right) cancers from TCGA are plotted according to the status of the *ALDH2* risk allele. P-values for significance between risk (+) and (-) are also provided (two-sided Mann-Whitney U test). **e.**

Frequency of mutations allocated to Sig. C in ESCC samples is plotted according to the status of alcohol drinking and smoking. According the history of heavy drinking and smoking, the subjects were divided into four groups; drinking (-) / smoking (-) (n = 18), (-) / (+) (n = 3), (+) / (-) (n = 40), and (+) / (+) (n = 96) as indicated in the panel. Boxplots represent the median, first quartile and third quartile with whiskers extending to the furthest value within 1.5×IQR and points show outliers.

Extended Figure 5. Mutations detected in single cell-derived colonies in WGS

VAF histograms of somatic mutations detected in 13 single cell-derived colonies analysed with WGS are depicted for each sample. Mutation number and driver genes mutated in each sample are indicated.

Extended Figure 6. Driver mutations in PNE samples

a. Distribution of mutations in 157 PNE and 519 ESCC samples is shown for driver genes significantly mutated in PNE or ESCC samples, including *NOTCH2*, *NOTCH3*, *CREBBP*, *FAT1*, *CHEK2*, *PAX9*, *EP300*, and *PIK3CA*. Mutations are depicted separately for PNE (top) and ESCC (bottom). **b.** Frequencies of driver genes in paired cancer/PNE samples from 68 ESCC patients. Genes in which mutations were observed in 5% or more samples either in PNE or ESCC were evaluated. Significantly differentially mutated genes ($q < 0.05$) between PNE and ESCC samples are indicated by asterisks. **c,d.** Number of common driver mutations (**c**) and maxMCFs (**d**) in each of high- and low-risk samples are plotted against subject's age according to sample size. Regression lines are provided with correlation coefficients and R^2 . P -values for significance of lifestyle ESCC risks are also indicated (one-sided Mann-Whitney U test). **e,h.** Mean±95%CI of standardized difference of mutation number (**e**) and maxMCF (**h**) between high- and low-risk samples are plotted for each sampling size and combined samples using a random effect model (RE model). P -value in the RE model is also indicated, together with the weight from each sample size (in %) for the model fitting (two-sided Wald test) (**Online Methods**). **f,g.** Mean±95%CI of standardized residuals of the number of indicated driver mutations (**f**) and their maxMCF (**g**) in high-risk samples against the linear regression model in low-risk samples (**Online Methods**) are plotted for 0.2 mm², 0.8 mm², and 4.0 mm² samples. Standardized residuals for combined samples using a random effect model are also shown (RE model). P -value in the RE model is also indicated, together with the weight from each sample size (in %) for the

model fitting (two-sided Wald test) (**Online Methods**). In the analyses of **c-h**, numbers of low- and high-risk samples in 0.2 mm², (0.8 mm²), and 4 mm² sampling are 34 and 19, (19 and 12), and 40 and 33 for **c,e,f**, 26 and 18, (13 and 12), and 30 and 31 for **d,h**, 5 and 5, (4 and 7), 8 and 19 for *TP53* in **g**, 14 and 18, (7 and 11), 21 and 28 for *NOTCH1* in **g**, 2 and 4, 2 and 9 for *PPM1D* in **g**, respectively.

Extended Figure 7. CNAs in PNE, dysplasia, and ESCC samples

a. Colour-gradient maps of CNAs and copy-neutral LOH as detected by SNP-array karyotyping or sequencing-based assays are shown for high- and low-risk PNE samples, as well as dysplastic tissues (n=12) and cancer samples (n=51) (middle panels). Fractions of genomes showing copy number gains (red), losses (blue), and copy-neutral LOH, or UPD (green), are also plotted (top panels). Information about histology, subject's age, sample size, as well as ESCC risks, are shown in top panels. **b.** Box plots of fractions of genomic regions showing CNAs in PNE (n=188), dysplasia (n=12), and cancer (n=45) samples; the median, first and third quartiles, as well as outliers, are indicated with whiskers extending to the furthest value within 1.5 of the interquartile range (IQR). *P*-values for significant differences are from two-sided Mann-Whitney U test. DP: Dysplasia. **c.** Effects of lifestyle ESCC risks and age on CNAs. Mean±95%CI of standardized residuals of the total fraction of genomic regions showing CNAs in high-risk samples compared with the linear regression model in low-risk samples (upper panel) and Fisher's z transformed correlation of age to the total fraction of genomic regions showing CNAs in low-risk samples (lower panel) are plotted for 0.2 mm², 0.8 mm², 4.0 mm², and 8 mm² samples, which are combined using a random effect model (RE model) *P*-value in the RE model is also indicated, together with the weight from each sample size (in %) for the model fitting (two-sided Wald test) (**Online Methods**). Numbers of low- and high-risk samples in 0.2 mm², and 0.8 mm² and 4 mm², (and 8 mm²) sampling size are 34 and 19, 19 and 12, 40 and 33, (and 3 and 28) and numbers of <50 y.o. and ≥50 y.o. samples from low-risk individuals are 20 and 14, 13 and 6, and 7 and 33, respectively. **d, f.** LOH maps of chromosomes 9 (**d**) and 17 (**f**) in PNE (top) and cancer (bottom) samples. Deletions and UPDs are shown by blue and green lines, respectively. Positions and mutation status of *CDKN2A*, *NOTCH1*, and *TP53* genes are indicated. **e, g.** Bar plots of frequencies of 9q UPD (**e**) and 17p LOH (**g**) in high- and low-risk samples. Individuals of <50 years of age

(low-; n = 40 and high-risk samples; n = 11) and those of ≥ 50 years (n = 56 and n = 81) were analysed. Number (n) of samples in each group is also indicated. *P*-values are for significant differences between both risk groups (two-sided Fisher's exact test). Horizontal bars indicate 95% confidence intervals from the binomial distribution.

Extended Figure 8. Spatial architecture of clones in representative PNE biopsies

a. Mutation analysis of 341 samples obtained by high-density sampling from 16 biopsy specimens from 14 individuals with different age, lifestyle ESCC risks, and ESCC status (bottom panels), using WES (249 from 10 biopsies) or targeted-capture sequencing of major drivers (92 from 6 biopsies). Total numbers of mutations (top panel) are depicted for samples analysed by WES. Shared mutations are shown in orange. Mutation status of major drivers and common CNAs are summarized (middle panels). **b-e.** MCFs of detected mutations are depicted for each sample obtained by high-density collection from PNE biopsies performed on low-risk healthy individuals in 20's (**b-d**) and an 81-year-old low-risk male (**e**). Four representative examples from 10 biopsies analysed by high-density WES sampling are shown. Mutations shared by a distinct set of samples or isolated samples are integrated to identify unique clones as shown in different colours. Driver mutations are indicated by down arrowheads. Right arrows indicate the clones in the same colour in **Fig. 4** and **Extended Fig. 9e**.

Extended Figure 9. Clones detected in high-density sampling

a-i. Spatial distribution of clones as revealed by WES or targeted sequencing of driver genes is depicted for biopsies from younger aged low-risk (**a,b**), middle aged low- and high-risk (**c,d**), aged (70 years) low-risk (**e,f**), and aged high-risk (**g-i**) subjects in a same manner as described in **Fig. 4**. For convenience, samples sharing discrete sets of mutations, as indicated by different colours, are summarized for four subjects (**a-d**), where mutations are frequently shared between distant samples, suggesting mosaicism. Sequencing platforms are indicated in each panel.

Extended Figure 10. Phylogenetic analysis of representative clones

Phylogenetic trees mapped to the location of samples are depicted for additional representative clones from a total of 29 clones seen in 5 biopsies from 5 elderly subjects. Imputed clonal structures are depicted in violin plots at the bottom, where

estimated frequencies (or prevalence) of imputed mutational clusters (vertical axis) in each sample are shown as the distribution of posterior probabilities (width of the violin plots) calculated from the Pyclone model. Colours correspond to those in the associated phylogenetic trees.

Figure 1

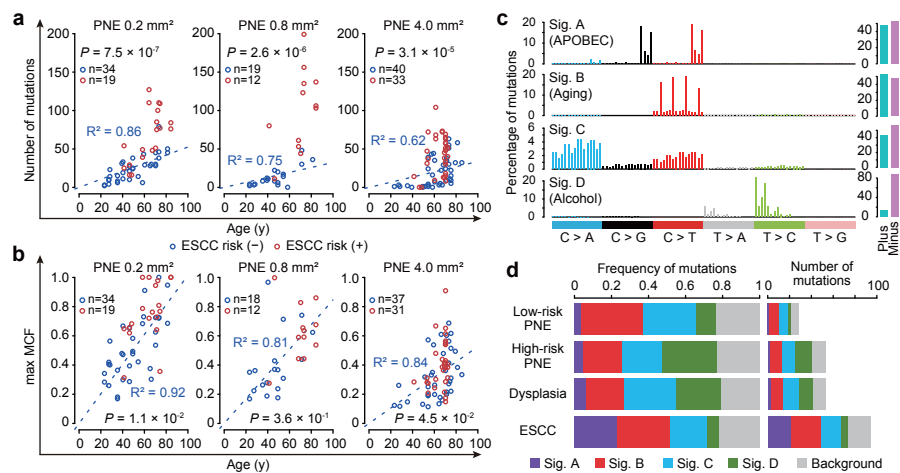


Figure 2

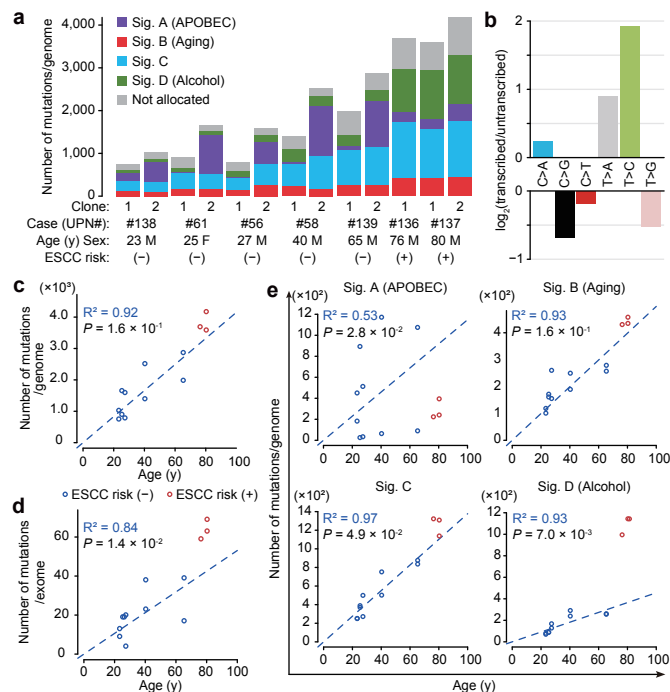


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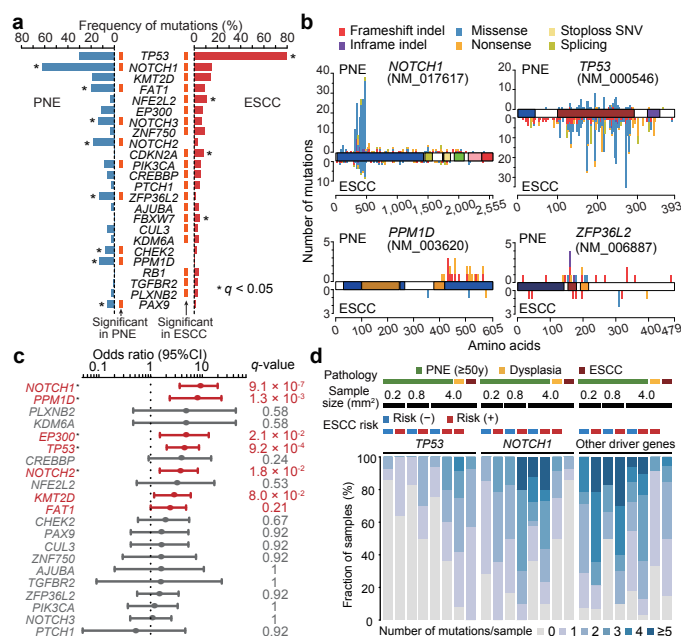


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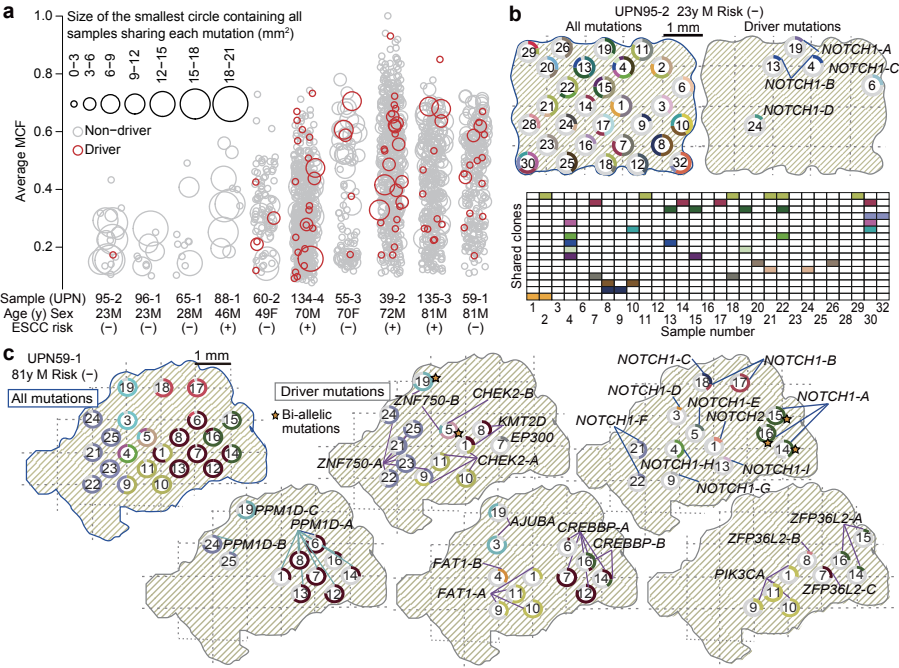


Figure 5

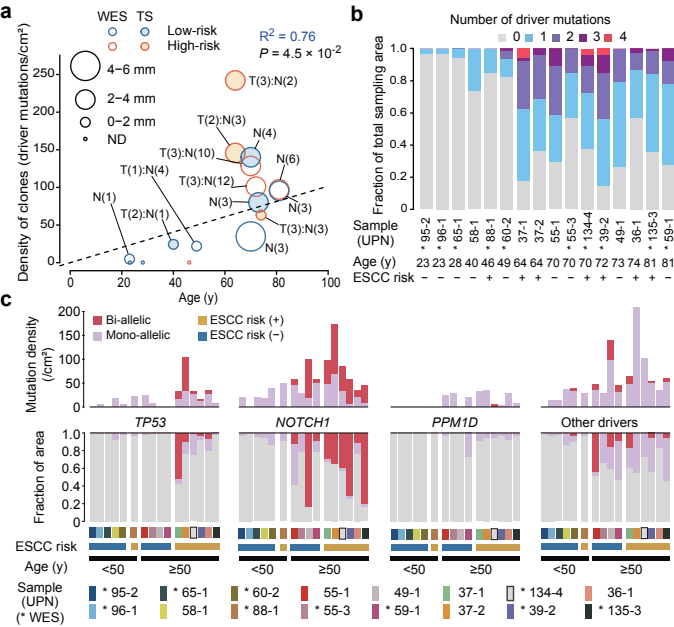
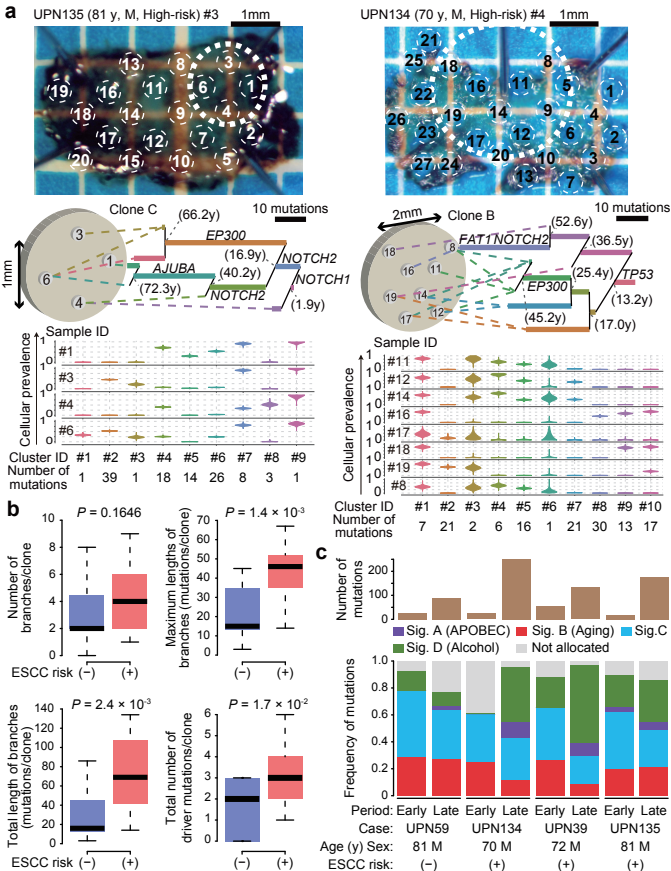
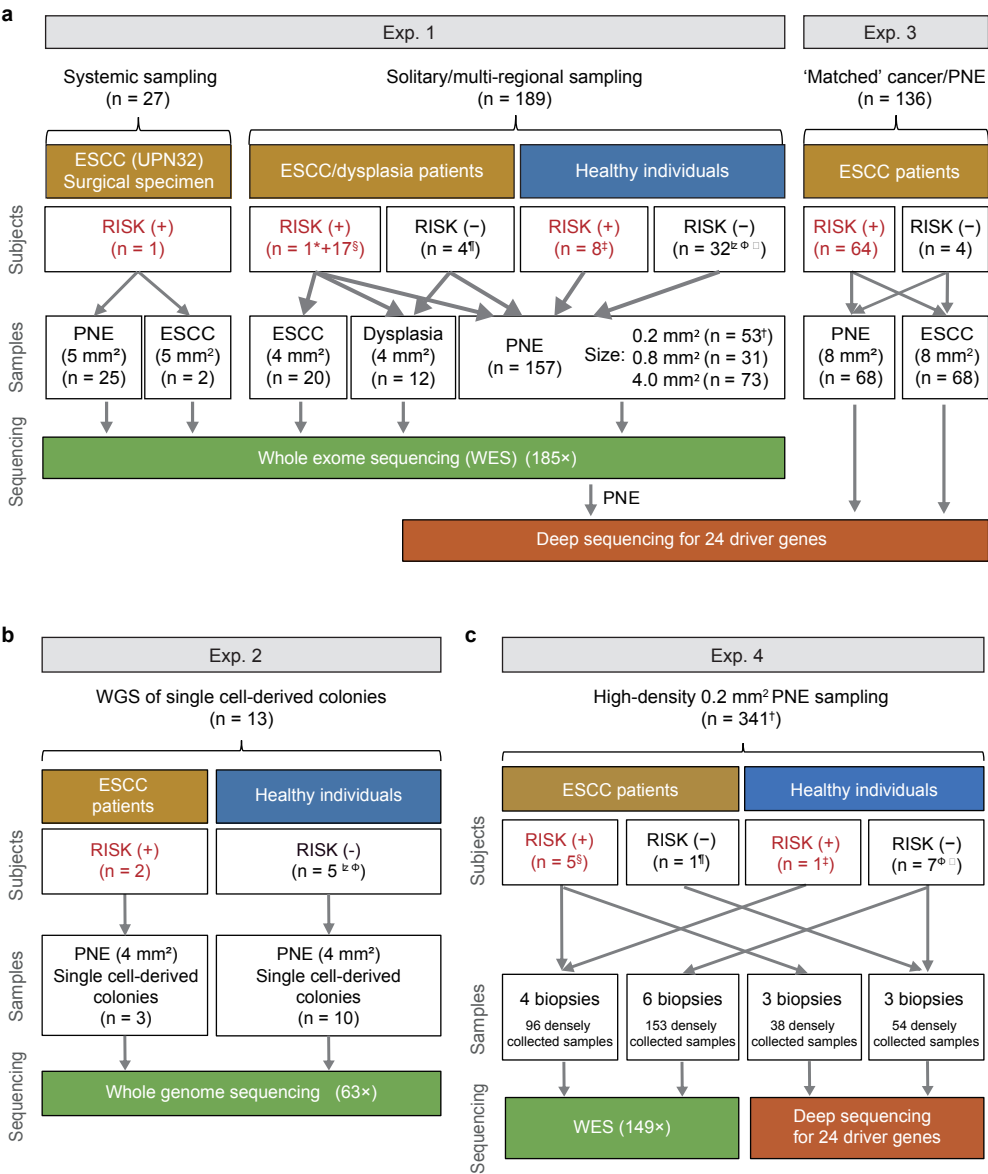


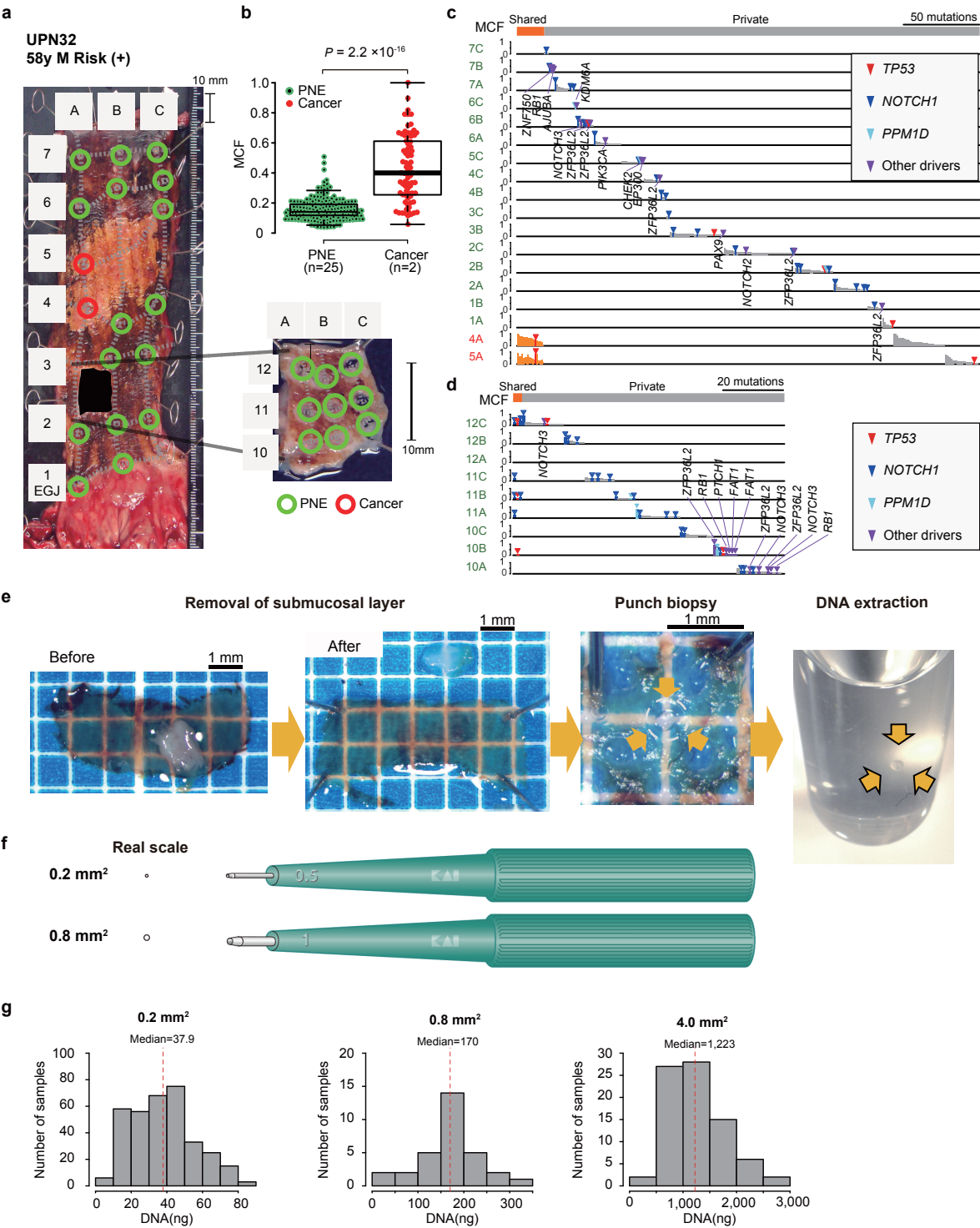
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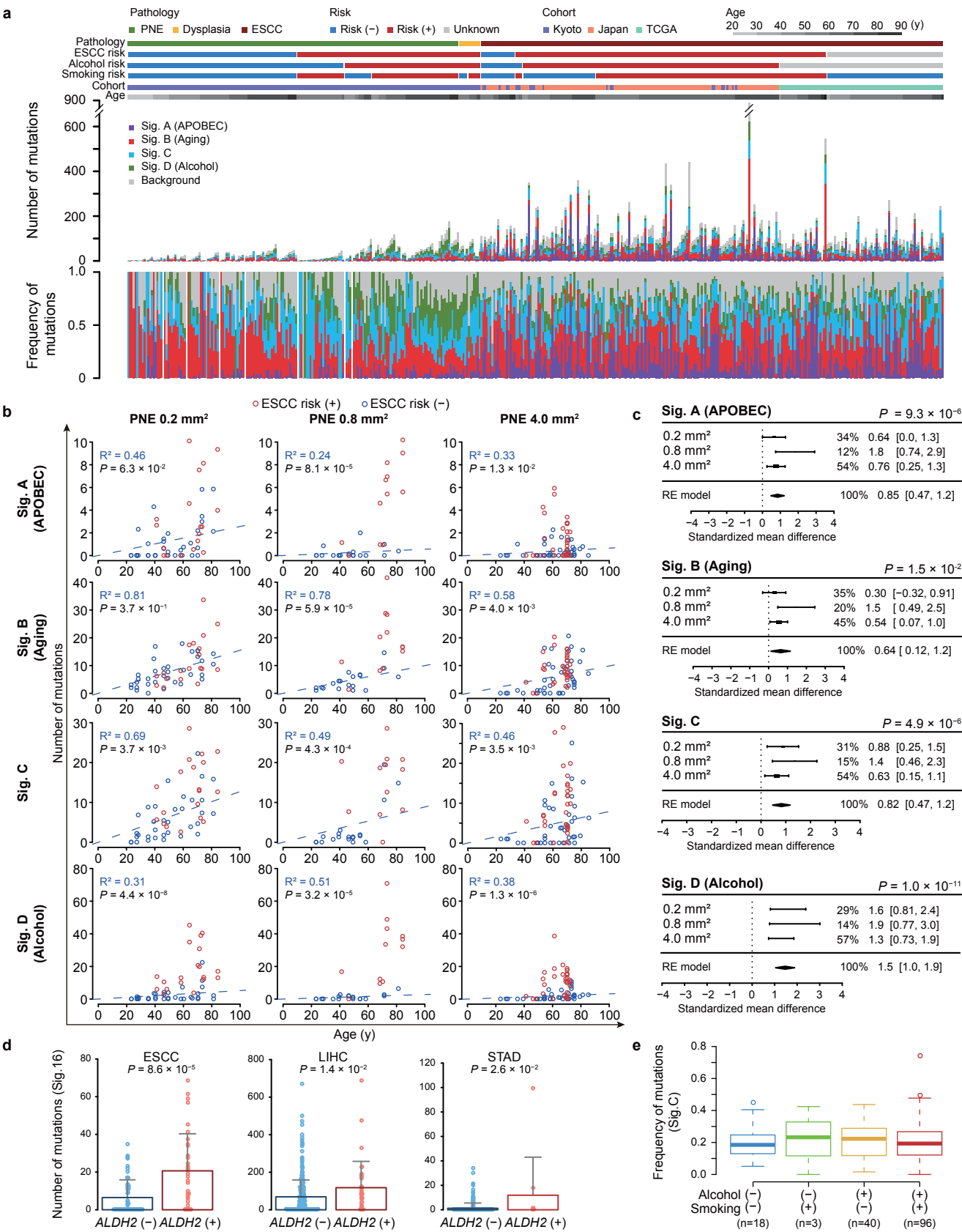
Extended Figure 1



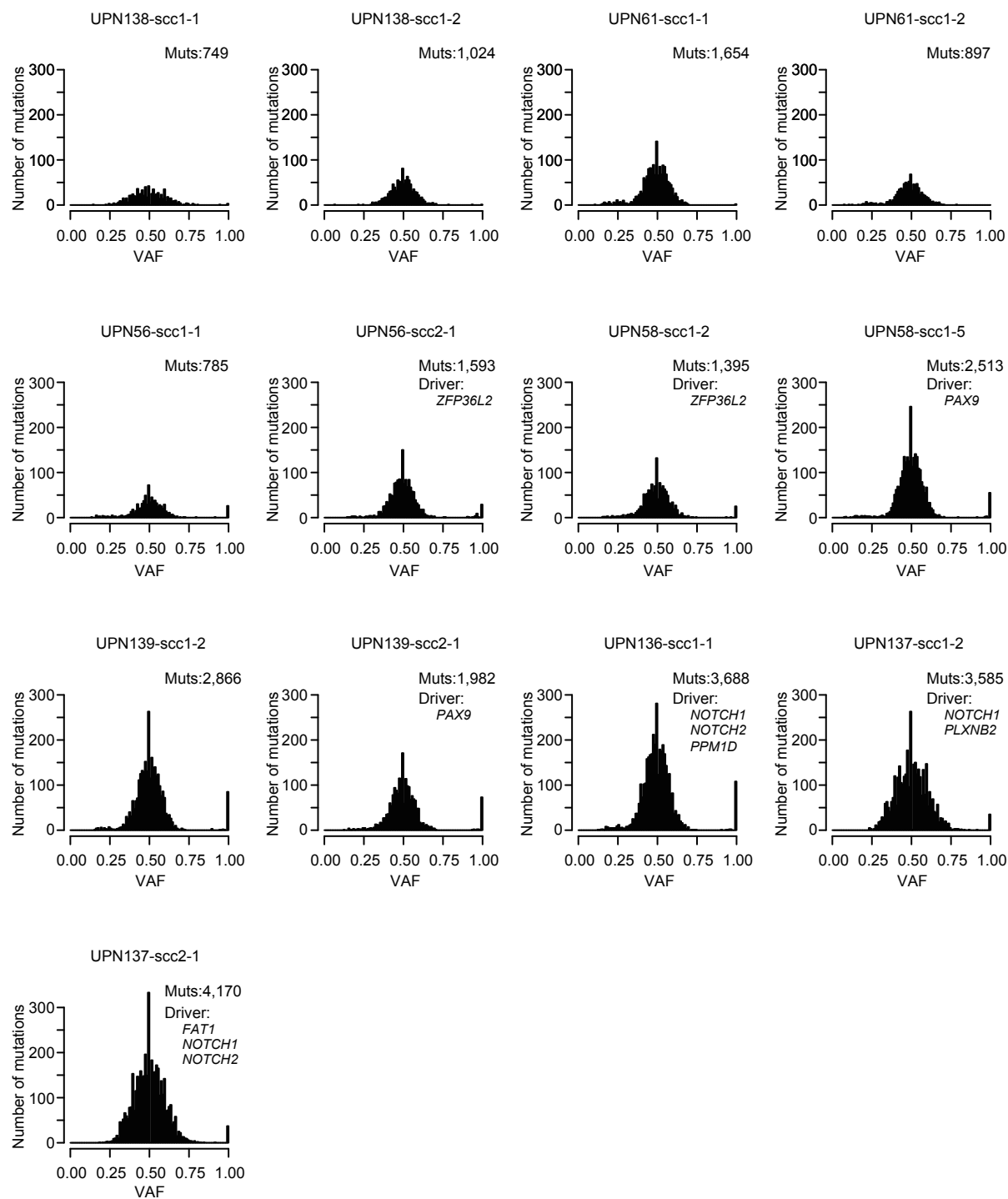
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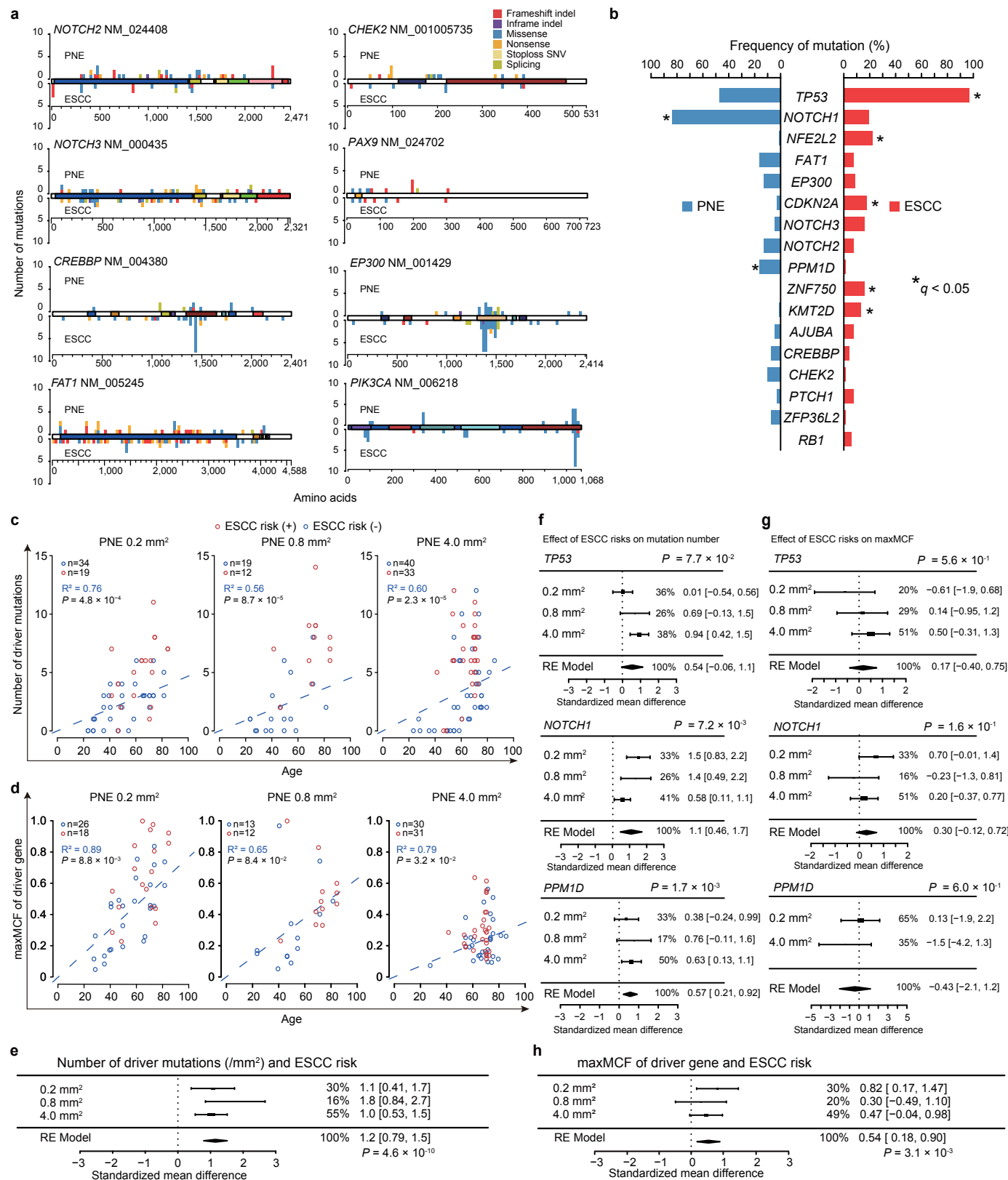
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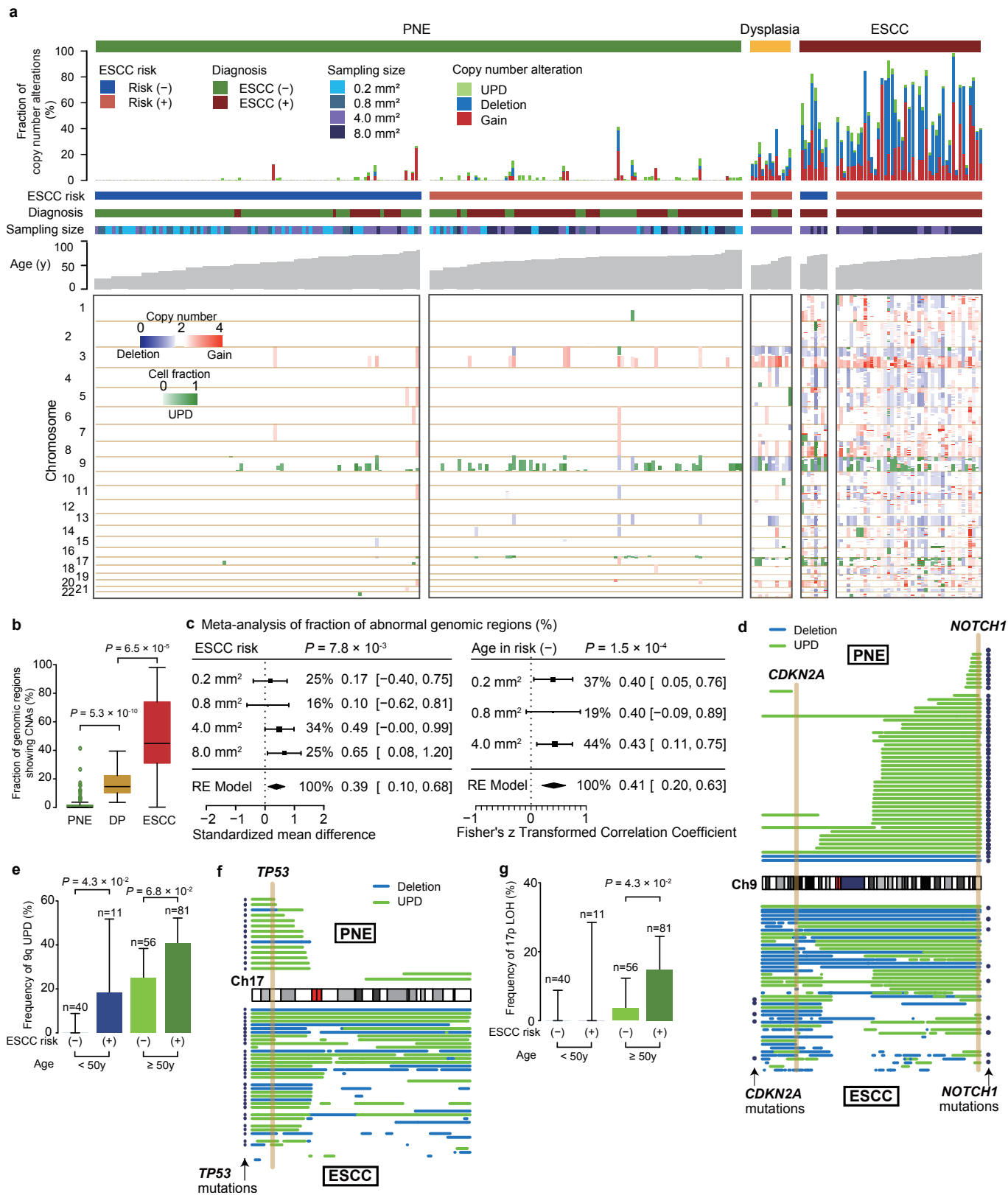
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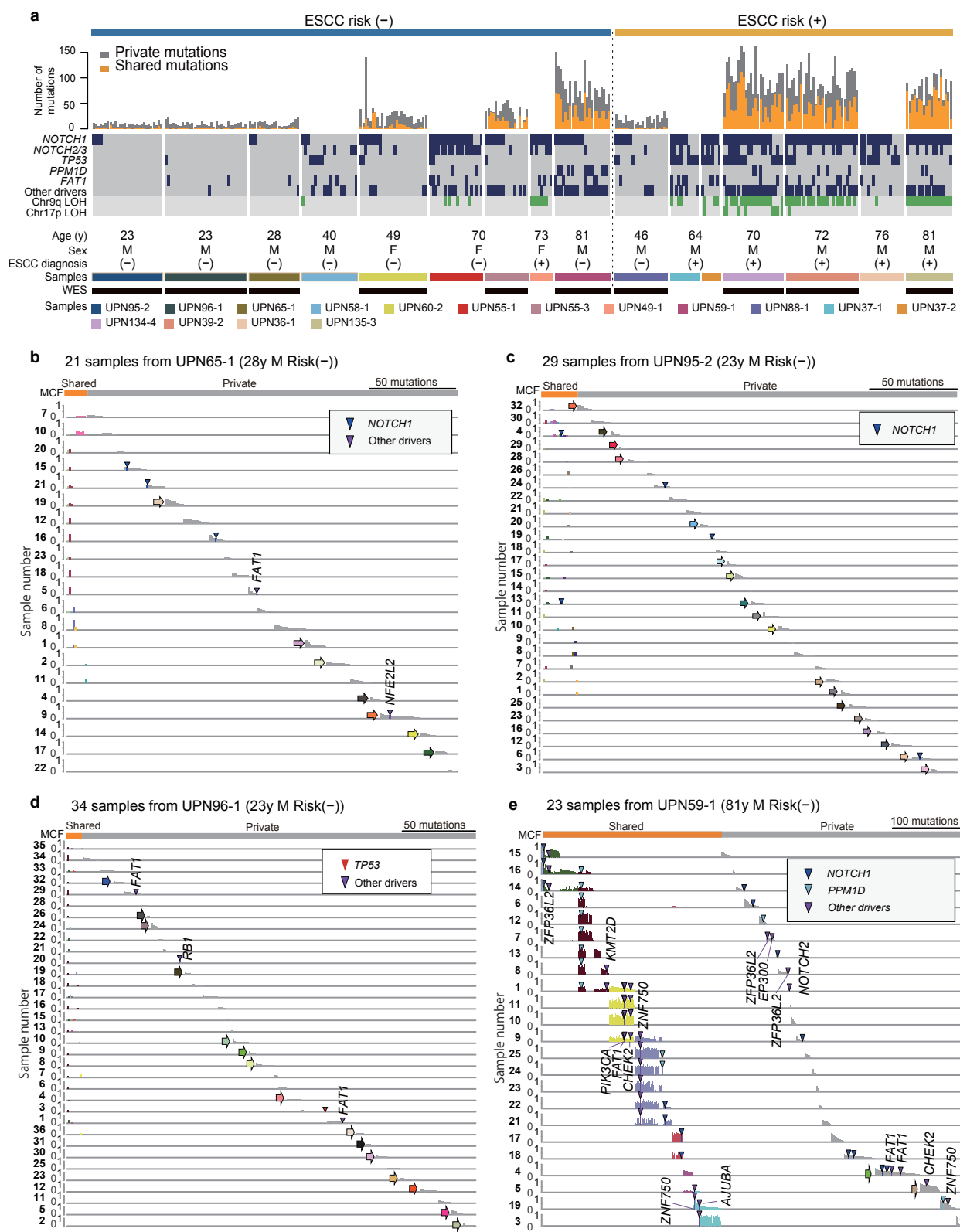
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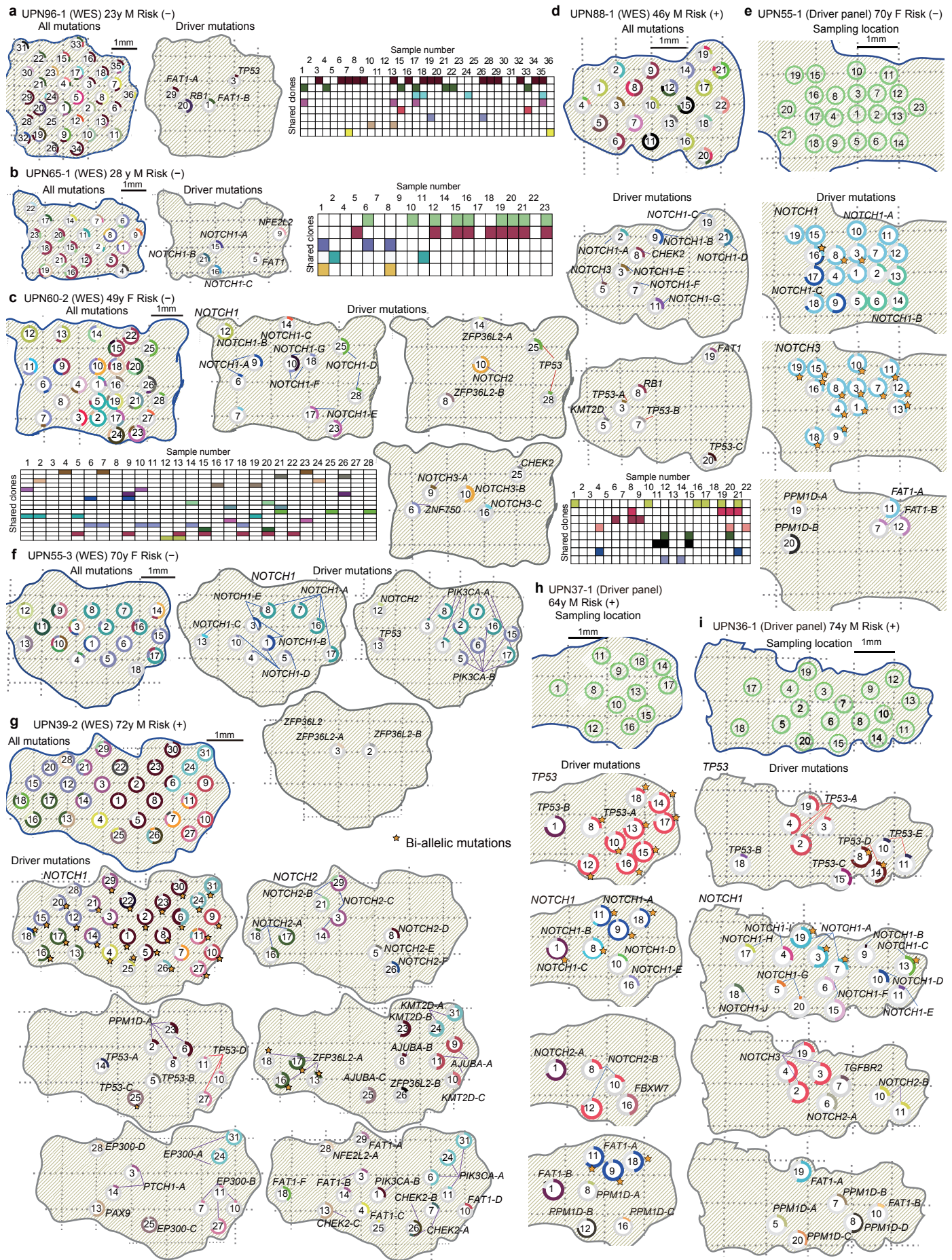
Extended Figure 7



Extended Figure 8



Extended Figure 9



Extended Figure 10

