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The complex nature of fragile site plasticity and its importance in cancer

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Common fragile sites (CFSs) are chromosomal regions characterized as hotspots for breakage and chromosomal rearrangements following DNA replication stress. They are preferentially unstable in pre-cancerous lesions and during cancer development. Recently CFSs were found to be tissue- and even oncogene-induced specific, thus indicating an unforeseen complexity. Here we review recent developments in CFS research that shed new light on the molecular basis of their instability and their importance in cancer development.

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Introduction

Common fragile sites (CFSs) are genomic loci characterized by gaps and breaks in metaphase chromosomes of cells grown under mild replication stress conditions that impede DNA synthesis [1]. They were first described in 1984 in human metaphase chromosomes of lymphocytes, but since CFSs have been identified in additional cell lineages [1,2,3••,4,5••]. CFSs are part of the chromosome structure and are present in all individuals. They differ from rare fragile sites, which are present in less than 5% of the population and are characterized by a specific nucleotide repeat expansion [6]. CFSs are conserved throughout evolution and orthologs of human CFSs have been found in syntenic regions of several mammalian species including mice and primates [7,8,9,10,11] and have also been described in yeast [12,13,14]. Currently, ~90 human CFSs are listed in the Genome Data Base (GDB); however, as will be discussed below, the repertoire of CFSs breakage (termed 'expression') depends on the cell type and the source of the replication stress. Here we review recent developments in CFS research to shed new light on the molecular basis of their instability and their importance in cancer development.

CFSs are hotspots for genomic instability, a hallmark of cancer, and can be found in precancerous cells [15,16,17]. CFSs are usually stable *in vitro* under normal growth conditions, whereas under DNA replication-stress conditions they are expressed. *In vitro*, replication stress can be induced by low concentrations of aphidicolin, an inhibitor of DNA polymerase, whereas *in vivo* replication stress may be generated by aberrant oncogene expression and is thought to exist in precancerous cells [18,19,20]. CFSs are found to be hotspots for sister chromatid exchanges and exhibit a high frequency of translocations and deletions in somatic cell hybrid systems [21]. Interestingly, CFSs are preferential hotspots for viral DNA integrations, which may lead to cancer development [22,23,24,25]. CFSs correlate with chromosomal breakpoints in tumors, and are involved in deletions of tumor suppressor genes and amplifications of oncogenes [26,27,28,29], however, many recurrent deletions in cancer remains unexplained [26]. As will be discussed below tissue-specific and stressor-specific factors may underlie yet unexplained fragile sites. Thus, understanding the molecular basis characterizing CFSs is of high importance for understanding chromosomal instability in cancer.

Characteristics and mechanisms underlying CFS instability

CFS expression under replication stress indicates that these regions fail to safely complete DNA synthesis during the S-phase. Certain CFSs fail to complete replication in the G2 phase and even in mitosis [30,31,32,33,34,35•]. Numerous mechanisms underlying the sensitivity of CFSs to breakage have been suggested, including late replication timing [30], origin paucity [36], replication fork arrest at AT-rich repeats [37,38], origin exhaustion under normal growth conditions [39], collisions between replication and transcription [40] and chromatin modifications [41] (reviewed in Ozeri-Galai *et al.*, 2014). More recently, it was further suggested that CFSs are enriched in cancer-related genes, microRNAs, binding elements and specific histone modifications that may also characterize fragile sites [42]. However, to date, no single mechanism can account for the sensitivity to replication stress across all the identified CFSs, which suggests that different characteristics or a combination of them form the basis for the enhanced sensitivity of CFSs to replication-induced DNA damage [42,43]. Since CFSs are unstable already in the earliest stages of cancer, it is

possible they possess a more profound role in tumorigenesis than merely participating in emerging genomic instability. In the past few years, the potential role of large genes and their transcription in CFS stability has been studied and is reviewed below.

It has been shown that many CFSs co-localize with very large genes, >600 kb long [44]. However, for most CFSs, there has been no comprehensive molecular identification analysis, and the breakage hotspots within a CFS do not necessarily co-localize with the large genes. Nevertheless, Le Tallec *et al.* (2013) showed that >80% of CFSs in the human genome harbor genes >300 kb long [3••]. This is a striking association given that the median length of human genes is ~20 kb and large genes only account for ~3% of human genes. Although the correlation between large genes and aphidicolin-induced CFSs is strong, it is not complete. It is also possible that very long non-coding RNA genes (lncRNAs) map to CFSs and have yet to be identified. Furthermore, not all large genes co-localize with CFSs, indicating that either not all CFSs have been detected or there are other characteristics underlying CFS instability.

One interesting question rising from the association between large genes and CFSs is whether their aberrations in cancer passively accumulate or actively contribute to tumorigenesis [45,46]. One possible cause for the co-localization of CFSs and large genes may lie in the functions of these genes as tumor suppressors (TSGs). Indeed, several CFSs have been shown to harbor TSGs, including *FHIT and WWOX* in FRA3B and FRA16D respectively [47,48]. In addition, a recent study of oropharyngeal squamous cell carcinoma identified a new group of six large genes located in CFSs which are down-regulated in tumors and thus were suggested as potential TSGs [49], supporting their role in tumorigenesis. However, many of the recurrent cancer deletions are hemizygous [26], supporting the hypothesis that alterations in CFSs are secondary events that do not contribute to the cancer development. Therefore, the relationship between CFSs and large genes may stem from their cancer-related function, but it remains to be further investigated.

It has long has been suggested that collisions between replication forks and the transcription machinery can lead to genomic instability [40,50]. In most of the genome, such encounters are avoided by the spatial and temporal separation of replication and transcription. However, a recent study of five CFSs associated with large genes (>800 kb) suggests that this separation is impossible in very long genes, because their transcription extends more than one cell cycle [40]. In such cases the replication and transcription machineries will inevitably meet on the same template. Accordingly, it was proposed that under normal conditions additional replication forks may help resolve collisions between transcription and replication complexes, but under replication stress transcription elongation is blocked, leading to the formation of DNA-RNA hybrids (R loops) which results in CFS instability. In line with the association between large genes and CFSs, it was recently shown that under replication stress conditions, copy number variations (CNVs) and CFS expression occur at the same loci, that are enriched in large active transcription units (TUs, >500 kb) [51•]. The authors argued that late replicating active TUs >1 Mb may be credible cell type specific predictors of induced CNVs hotspots and CFSs. These results suggest that CNVs and CFSs are different manifestations of the same mechanism driven by late replicating-large TUs, and support the proposed role of active transcription of large genes in CFS instability [51•]. The authors proposed a model in which active transcription of late replicating loci increases failure of origin firing leading to unreplicated DNA, resulting in activation of a replication error-prone mechanism after S-phase. Overall, these studies indicate that active transcription of large genes may impair CFS stability via a transcription-dependent mechanism. However, recent reports have challenged these conclusions by showing no correlation between the transcription of large genes and CFS fragility [3••,52••]. It should be noted however that different approaches were used in these reports for measuring transcription which may underlie the differences. Therefore, the role of active transcription *per se* in FSs instability remains to be comprehensively explored.

Recently, a new group of recurrent fragile regions was reported [53]. These are early replicating fragile sites (ERFSs) identified by genome wide localization of DNA repair proteins in B lymphocytes under replication stress conditions. This indicates that their stability depends on faithful replication. Moreover, ERFSs co-localize with highly expressed gene clusters and are enriched in repetitive elements, indicating that 'difficult to replicate regions' as well as replication-transcription collisions may lead to genomic instability even in non-late replicating regions. It would be worthwhile to further investigate the mechanisms impeding the faithful repair of such regions. Altogether, CFS instability is caused by different mechanisms and their combinations which may differ between cell types and as the result of different stressors.

CFS plasticity

Tissue plasticity

It has been shown that the repertoire of CFS instability is tissue specific [2,3••,4,5••]. Le Tallec *et al.* (2011) analyzed the landscape of CFSs in fibroblasts and lymphocytes and found a different repertoire of fragility. Only two CFSs (*FRA3B* and *FRA16D*) were expressed in the different cell types; however, their level of expression substantially differed. In a later study, Le Tallec *et al.*

(2013) mapped CFSs in epithelial and erythroid cells and found that less than 20% of CFSs were shared between the different cell types. Hosseini *et al.* (2013) also found a distinct pattern of fragility in epithelial cells that differed from that in lymphocytes and fibroblasts [5••].

One mechanism that may underlie the cell-type specific repertoire of fragility is the tissue-specific replication timing program. Analysis of several cell-type specific CFSs using Repli-Seq found different replication timing patterns at these sites. This analysis supports the origin paucity model, in which the density of replication initiation events is decreased at expressed CFSs and is regulated in a tissue-specific manner [4,5••]. Another possible explanation for cell-type specific fragility is epigenetic differences between cell types. Little is known about histone modification profiles in CFSs; one study found hypoacetylation in 5/6 analyzed CFSs, which implies condensed chromatin structure at these sites that may impair DNA damage repair [41]. In light of the tissue specificity of CFS expression, it would be intriguing to investigate epigenetic features that may underlie CFS instability in different cell types.

Stress-induced plasticity — the effect of cancer genes

Recurrent instability in cancer can only be partially explained by CFS instability or structural rearrangements in cancer genes. A large scale analysis of ~750 cancer cell lines revealed that recurrent deletions occur preferentially in genomic regions harboring a tumor suppressor gene or in CFSs [26]. However, the molecular basis for the majority of recurrent deletions remains unexplained. Interestingly, the analysis revealed an instability signature in CFSs characterized by small hemizygous deletions which was also found in many 'unexplained' regions, as no association with known aphidicolin-induced CFSs or recessive cancer genes was found [26].

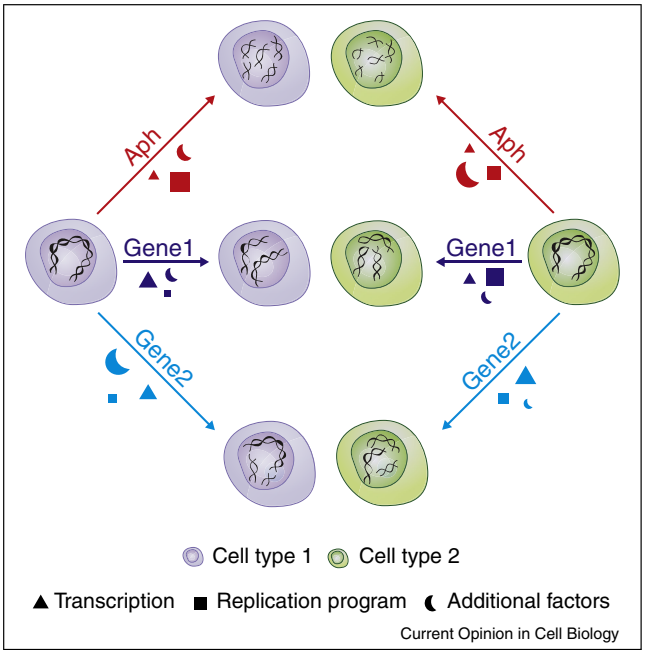
The aberrant expression of oncogenes generates replication stress [18,19,20]. Therefore, Miron *et al.* (2015) investigated whether oncogene-induced replication stress induces fragile site (FS) expression. Overexpression of cyclin E or mutated H-Ras in normal human fibroblasts indeed resulted in FS expression. Unexpectedly, a unique fragility repertoire was found for each oncogene which differed from the aphidicolin-induced fragility repertoire in the same cells. Strikingly, 70% of the identified FSs showed condition-dependent expression and ~50% were induced solely by oncogenes. Similar to aphidicolin-induced FSs, almost all of the FSs induced by cyclin E or H-Ras co-localized with at least one large gene. However, transcription analysis of several large genes residing in oncogene- or aphidicolin-specific FSs revealed no correlation between transcription and fragility, although a comprehensive analysis has yet to be performed. Interestingly, most of the cyclin E as well as the H-Ras-induced FSs coincided with recurrent deletion clusters previously defined by Bignell *et al.* (2010) as 'unexplained deletion clusters' [52••]. These findings highlight the complexity of recurrent fragility in cancer cells, since different oncogenes may create a unique fragility landscape even in the same cell type. Additionally, cyclin E overexpression in epithelial cells induced loss of genomic regions [33] that poorly overlap with cyclin E-induced FSs in fibroblasts [52••]. These regions are located in late replicating domains and some overlap with aphidicolin-CFSs and are characterized by replication origin paucity and unusual DNA structures. This suggests that oncogene-induced instability depends on the combination of the specific oncogene and the cell type (illustrated in Figure 1).

CFS replication beyond the S-phase

Many proteins have been reported to play a role in CFS stability maintenance, including DNA damage checkpoint proteins, DNA damage transducers, mediators, and DNA repair proteins (reviewed in Ozeri-Galai *et al.*, 2014). The importance of these proteins in maintaining CFS stability supports the role of fork stalling and under-replicated DNA in CFS expression, as some of these proteins function in inhibiting fork stalling and their depletion decreases replication fork progression. Under replication stress conditions CFSs were shown to be under-replicated loci [30]. Furthermore, there are studies showing activation of the network of proteins required for replication-induced DNA damage repair (such as ATR), yet the cells enter mitosis with under-replicated DNA expressed as CFSs [54]. This relates to some of the most interesting unresolved issues addressed in recent years: how do cells enter mitosis with under-replicated loci and what is the fate of these cells. Koundrioukoff *et al.* (2013) suggested that limited activation of the ATR pathway prevents fork collapse while permitting mitotic onset with under-replicated loci and CFS expression [54]. The fate of cells with under-replicated regions was recently addressed. Two reports found that MUS81-EME1 and ERCC1, DNA structure specific nucleases, localize to CFSs in early mitosis and promote their processing, thereby enabling the resolution of sister chromatids and subsequent chromosome segregation [55,56]. Thus, this late intermediate processing could be responsible for the phenotype of CFSs in metaphase chromosomes [55,56].

Importantly, DNA synthesis at CFSs was shown to occur during mitosis, as indicated by EdU foci in metaphase chromosome gaps or breaks [35•]. Mitotic DNA synthesis depends on POLD3 and is promoted by MUS81-EME1 activity. Inhibition of mitotic DNA synthesis was shown to lead to chromosome mis-segregation and non-junction, increasing ultrafine anaphase bridges, but reducing CFS expression. Thus, such loci may not replicate faithfully even in mitosis and may be the source of genomic instability. Co-localization of CFSs with DNA synthesis

Figure 1



A model for the molecular basis of tissue-specific and stressor-specific landscape of recurrent replication-induced chromosomal fragility. The repertoire of FSs induced by a single stressor only partially overlaps between different cell types. Furthermore, the fragility repertoire induced by chemicals (aphidicolin) or aberrantly activated cancer genes (oncogenes) differs within the same cell type. Alterations in transcription, replication program or in yet unknown factors, their relative contribution and possible different combinations, may be cell-type and/or stressor-specific. These alterations are likely to underlie the difference in sensitivity of genomic regions to replication-induced fragility. The size of the symbols representing transcription, replication program and additional factors, reflects their relative contribution to fragility.

in mitosis may imply that the gaps and constrictions on metaphase chromosomes represent replication loci rather than DNA breaks.

However, mitotic replication might be induced by DNA lesions. Works have reported DNA damage markers and translesion synthesis at CFSs [34,57,58]. The DNA damage response proteins γH2AX and DNA-PKcs were found on the majority of expressed fragile sites in metaphase chromosomes [57], indicating the existence of DNA damage at these sites. Additionally, CFS processing was found to depend on translesion synthesis polymerases (TLSs) [34,58], thus further indicating DNA damage at CFSs in mitosis. Depletion of polη, a TLS polymerase, increases CFS expression and EdU foci in mitotic cells; hence indicating that polη plays a role in CFS replication prior to mitosis. This type of replication reduces under-replicated DNA in mitosis [58]. Moreover, REV3, a subunit of polζ, was found to be expressed in G2/M and its depletion led to an increase in anaphase bridges and CFS expression, indicating that REV3 plays a role in CFS replication in G2/M [34]. Thus, CFSs on metaphase chromosomes probably represent mitotic DNA replication as well as repair of DNA damage. Importantly, cells with under-replicated loci were shown to proceed from mitosis to the G1 phase, in which a further attempt to repair the damage was demonstrated [59]. However, further studies of the fate of these cells and their potential contribution to genomic instability driving cancer development are required.

Concluding remarks

Replication impairment underlies CFS instability. Incomplete replication and/or damage repair at CFSs may result in their sensitivity to breakage, which can lead to chromosomal mis-segregations, CNVs and chromosomal rearrangements that can drive cancer development. At present, the molecular mechanism underlying the majority of recurrent cancer alterations is unclear. Recent advances have highlighted the complexity of CFS expression and reinforce that a better understanding of their instability can contribute to our understanding of genomic instability in cancer. Since no single mechanism can account for the fragility of different FSs, it is reasonable to assume that tissue-specific crosstalk between features/mechanisms induced by different sources of replication stress underlies the sensitivity and instability found in cancer. Therefore, when investigating the mechanism underlying recurrent chromosomal instability in cancer, the cell type specific FS repertoire and the source of replication stress should be considered. However, such data are still to be discovered. In this regard, a high throughput approach to identify FSs induced by different replication-stressors, is required. One such promising approach, which to date has only been applied in yeast, uses a newly developed methodology termed break-seq [60] to identify replication-induced breaks. In light of the recently identified FS plasticity, a comprehensive analysis of the factors affecting instability is required to better understand genomic instability in cancer.

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