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Open Sesame: activating dormant replication origins in the mouse immunoglobulin heavy chain (Igh) locus

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Abstract

Chromosomal DNA replication in mammals initiates from replication origins whose activity differs in accordance with cell type and differentiation state. In addition to origins that are active in unperturbed conditions, chromosomes also contain dormant origins that can become functional in response to certain genotoxic stress conditions. Improper regulation of origin usage can cause genomic instability leading to tumorigenesis. We review findings from recent single-molecule DNA fiber studies examining replication of the mouse immunoglobulin heavy chain (Igh) locus, in which origin activity over a 400 kb region is subject to dramatic developmental regulation. Possible models are discussed to explain such differential origin usage, particularly during replication stress conditions that can activate dormant origins.

Investigation of the initiation of eukaryotic DNA replication over the past few years has clarified the composition of the machinery that causes an origin to fire, that is, to initiate the formation and outward movement of two replication forks. Less well understood is how the activity of this machinery can be regulated by the differentiation state of the cell, such that particular chromosomal regions display origin activity only in certain cell types. Furthermore, use of genotoxic stress conditions has been found to trigger the firing of so-called dormant origins. In this review, we provide a brief overview describing possible models to explain how the activity of an origin might be controlled, focusing on the mouse Igh locus.

Unlike replication in *S. cerevisiae* and that occurring in specialized elements in higher eukaryotes [1], mammalian chromosomal DNA appears to be primarily initiated from zones in which origin activation can occur at many inefficient sites within particular 50-500 kb regions. It should be stressed that while there may be more than one initiation site in a particular zone in a single cell, these initiation events occur at different sites within this zone in different cells. Because multiple origins can stochastically fire in each zone [2], determination of the number of mammalians origins is a challenging exercise, but it has been estimated that 30,000 to 50,000 origins fire each cycle in unperturbed conditions. This compares to ~400 origins that become activated in budding yeast [3]. Mammalian replicons have been estimated to average from 100 to 500 kb in length [4]. Although this potentially

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yields a smaller number of replicons relative to origins, this difference could be at least partly explained by the firing of several origins (termed replicon clusters) in the initiation zones of most replicons. Replication of multiple replicons occurs in ‘factories’ that are visible as punctate nuclear foci after immunological staining for replication proteins. The nuclear locations and size of replication foci have somewhat distinctive patterns at different stages of S phase.

Replicon size and origin location differ dramatically with the differentiation state of the cell. Genome-wide analysis of replication timing shows that, during differentiation of mouse embryonic stem (ES) cells to neuronal precursor cells, smaller replicons become consolidated into larger replication domains [5]. Such studies indicate that differentiation causes a global reorganization of origin usage, a transformation also detected during development of early Xenopus embryos [6]. Origin utilization is also highly regulated in response to genotoxic stress, with one potential consequence being the activation of normally silent (dormant) origins. For example, investigation of Chinese hamster cells by the Debatisse laboratory demonstrated that a reduction in nucleotide pools by hydroxyurea (HU) treatment modulates origin usage and caused the firing of dormant origins at particular sites [7,8]. Recent examination of origin location in human cells found that HU treatment led to marked increase in dormant origin firing, often found in the vicinity of transcriptionally-active genes [9].

Another important regulatory control over replication initiation is origin timing, that is, the time in S-phase in which a particular origin fires. One major factor governing origin timing is chromatin structure, with genome wide studies showing that regions with histone modifications that mark active genes generally have early firing origins, while origins in regions with repressive modifications reflective of heterochromatin-rich DNA usually fire late in S-phase [10]. These findings are consistent with studies showing that early origins are preferentially located near highly-expressed genes, while late origins are distant from transcribed genes [11]. It is perhaps not surprising that replication timing is strongly affected by differentiation state, cell type, and even gender of the organism from which the cells were derived [12,13], with at least half of the human genome showing a tissue-specific variation in replication timing [14]. Because origin choice under either unperturbed and stress conditions can potentially impact tumor suppression [15] and the stability of common fragile sites [16], determination of where origins are located and how origin firing is regulated are both biologically-important and clinically-relevant goals.

The importance of determining origin location has led to the development of multiple methods that can identify origin activity on chromosomal DNA. Current approaches often entail genome-wide analysis that map the positions of origin-selective molecular markers including: nascent single-stranded DNA (ssDNA) in the ~0.5 to 1.5 kb size range (i.e., longer than Okazaki fragments [17]; single-strand template regions [18]; sequences replicated immediately after S-phase onset [19]; and DNA fragments containing replication bubbles [20] (reviewed in [21]).

An alternative approach to studying origin activation focuses not on whole genome analysis, but rather on the detection of origin firing at particular chromosomal regions, using single-molecule DNA fiber methods, such as SMARD (single-molecule analysis of replicated DNA; [22]). The use of SMARD and related single-molecule approaches provide a powerful combination of relatively high-resolution origin mapping (i.e., to within 5 kb) and a large data set (i.e., number of individual DNA molecules) for the chromosomal region of interest. Such techniques therefore provide the opportunity to examine the influence of novel sequence elements, altered chromatin structure, genotoxic stress, and other variables on the replication activity of specific metazoan origins.

The differential activation of DNA replication origins in the murine Igh locus has been studied in considerable detail using SMARD (see [22,23]). In mouse embryonic stem (ES) cells where the Igh locus is transcriptionally-inactive, replication forks initiate in a zone whose proximal boundary, relative to this locus, is ~80 kb downstream from the Cα gene. After initiating in the zone, replication of the Igh locus then occurs by passage of a single fork that extends for up to 400 kb, and potentially for more than 4.5 hours, before terminating. We have called this a temporal transition region (TTR; [22]). SMARD analysis of a 180 kb SwaI segment containing most of the Igh constant region genes detected no initiation events in more than 600 replicated molecules in several different experiments (Fig. 1A). Attempting to induce origin activity by perturbation of transcription or local acetylation of the SwaI segment was ineffective [23], indicating that this region is highly refractory to origin activation in ES cells. If dormant origins are present in the Igh constant region in mouse ES cells, they are not easily activated by localized modifications. Work by the Debatisse laboratory has also suggested that histone acetylation does not modulate origin activity [24].

In mouse proB cells, remarkably, origins within the SwaI segment can be easily activated. SMARD examination of replicated molecules containing this segment detected more than 50% with initiation events (Fig. 1B; [22]). One or two origins were found in each of the SwaI segment molecules, although the position of the origin(s) appeared somewhat random in different DNA molecules, and hence in different proB cells. Additional initiation events may have occurred that resulted in long forks that had proceeded off the end of the SwaI segment, hence the initiation is no longer visible as a red patch surrounded by green. It is possible that the origins that fire in proB cells are either inactive or present in a dormant state in ES cells, and in other cells in which the Igh region is transcriptionally silent.

In an attempt to activate origins in the ES SwaI segment, a 10.7 kb DNA element obtained from the aforementioned downstream initiation zone was inserted into the segment [23]. In contrast to the lack of effect seen in ES cells by altering the chromatin modification state or inducing transcription in this region, insertion of the downstream element was found to be partially successful in inducing new initiation events within the SwaI segment. Somewhat surprisingly, the newly activated origins were not located in the inserted element, but rather in adjacent sequences (Fig. 1C). These novel origins may be among those that are normally active in proB cells, or may instead represent dormant origins that can become activated because of the presence of the new sequence. In the latter case, the dormant origin within the Igh locus of ES cells represents a class of origin that cannot be easily activated, in that only a relatively few initiation events were found.

Our favored hypothesis to explain these results postulates the existence of several types of origins that could be classified as either dormant or very inefficient. Although we use the term dormant below, we will extend the definition to include those origins that fire at very low efficiency. One class of these normally unused origins can be activated by DNA sequence insertions within a locus. A second class of dormant origins could be cell type-dependent, such that the origin is completely inactive in one cell type but active in another cell type because of different gene expression patterns. Another type of dormant origin could be activated by replication stress. These three classes of dormant origins are united by their ability to be activated under particular cellular conditions.

Even though they only rarely fire under normal conditions, current evidence indicates that dormant origins are generated using the same general machinery that forms pre-replication complexes (pre-RCs) at normal origins. Briefly, pre-RC formation in metazoan cells generally proceeds from a bound origin recognition complex (ORC) which serves as a scaffold that supports loading of replication licensing factors Cdc6, Cdt1, MCM9, and

MCM2-7 (Figure 2). Following origin firing, the hetero-hexameric MCM2-7 complex, in combination with Cdc45 and the GINS complex, serves as the replicative helicase [25]. A recent study has provided evidence that Cdc45 is limiting for replicon usage in mammalian cells [26].

What are the molecular differences that might cause the differential ability of an origin complex to fire only rarely, selectively in particular cell types, or in response to stress? Because ORC is required for formation of the pre-RC complex, it is possible that the origin activity of a particular DNA sequence could lie in the efficiency of ORC binding or association (Figure 3A). Thus, the lack of origin activity in the SwaI segment of ES cells may be caused by inefficiency of ORC binding (top of panel A). In the case of the DNA insertion causing an increase in origin firing in the SwaI segment, this activation could be due, for example, to a novel DNA element that supports the binding of protein factors that more efficiently recruit ORC (second line of panel A). Other obvious possibilities are that the inserted DNA causes changes in the nuclear location (e.g., see [27]), or chromatin modification state of the loci (e.g., see [28]), again with the end result that ORC binding is facilitated and origin activity is increased. An alternative to this model is that, while ORC could weakly associate with the inserted sequence, it would nevertheless be able to activate dormant origins at distal sequences by looping of the intervening chromosomal DNA (third line of panel A). The high level of origin activity in the proB SwaI segment may be a result of a high level of ORC binding (bottom line of panel A), potentially resulting from increased transcription in the SwaI segment, altering the chromatin structure of the transcribed sequences and thereby improving ORC association.

A second possibility to explain differential origin activity focuses not on ORC binding, but rather on the ORC-mediated loading of the MCM complex. This model is best explained in the context of DNA replication stress. Past examination of the relative amounts of replication licensing factors associated with chromatin led to the initially confusing observation that the MCM complex is present at a ~20-fold molar excess compared to ORC [29-32]. A role for the excess MCM is suggested from studies using Xenopus extracts depleted for particular MCM subunits. In the absence of stress, the depleted extracts were seen to support a similar density of origins as non-depleted extracts. When aphidicolin (an inhibitor of the replicative DNA polymerases) was added to cause replication stress, the non-depleted extracts showed a 5- to 10-fold increase in origin density, while the depleted extracts had no apparent increase [33]. Such studies have led to the postulate that, after ORC-mediated chromatin loading, the excess MCM complexes have the potential to activate origins under stress conditions. Such a model is consistent with studies indicating that ORC and Cdc6 are not necessary for origin activity after MCM loading has taken place [34]. MCM complexes on chromatin show relatively non-specific localization (e.g., see [35]), suggesting that diffusion of MCM complexes away from the site of loading provides the potential for ORC-distal regions to act as origins under stress conditions.

To further clarify, replication origins are subject to stress-dependent controls in addition to those described above. Late-firing and dormant origins are also repressed by the action of checkpoint kinases under genotoxic conditions. Chk1/Rad53, an effector kinase downstream of the ATR/Mec1 checkpoint kinase, has been shown to inhibit the activity of such origins in a variety of eukaryotic systems [18,36-43]. Inhibition of ATR and ATM signaling in otherwise unperturbed *Xenopus* egg extracts has also been demonstrated to lead to a 2- to 3-fold increase in the density of active origins [44,45], indicating that sustained, albeit low-level activation of the ATR/Chk1 pathway during a normal S-phase causes the inhibition of late origin firing. Recent evidence indicates that, while dormant origin activation in late-firing chromosomal regions is subject to Chk1-mediated suppression, origins in pre-existing replication factories are refractory to this inhibition [46]. Paradoxically then, increased firing

of dormant origins can be seen under conditions that cause checkpoint activation and the subsequent repression of late-origin firing. What causes the observed increase in dormant origin firing seen under replication stress conditions? Various solutions have been hypothesized to explain these contradictory effects (e.g., [33,47-49]), including the possibility that the stalled replication fork generates a signal that stimulates origin firing in the vicinity of the stall. A variation of this theme is that fork stalling increases the window of time in which a downstream origin is allowed to fire because non-dormant origin firing is generally a stochastic process [50].

That MCM complexes can serve as origins subsequent to ORC-mediated loading can also apply in non-stress conditions (Figure 3B). In this model, a highly-active ORC complex that loads more MCM onto DNA would be expected to generate a region with a higher origin activity (bottom of panel B), relative to a region containing a weakly-active ORC complex (top of panel B). Note that this model does not preclude the involvement of other factors to provide origin activity in combination with the MCM complex (e.g., Cdc45 and GINS), but rather that ORC is dispensable after loading MCM. Similar to the ORC occupancy model, ORC activity could be modulated by features of the DNA site, including the binding of factors that directly associate with ORC, potentially stimulating ORC activity (middle of panel B). This mechanism could therefore not only explain the ability of the Igh insertion site to provide higher origin activity, but also to cause origin activity at sites distal from the insertion, as detected by SMARD. It should be emphasized that the ORC occupancy and ORC activity models discussed above and in Figure 3 are not mutually exclusive. Indeed it would be surprising if future studies did not find that both types of regulation, and others yet to be identified, were employed in regulating origin activity in metazoans.

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•• of outstanding interest

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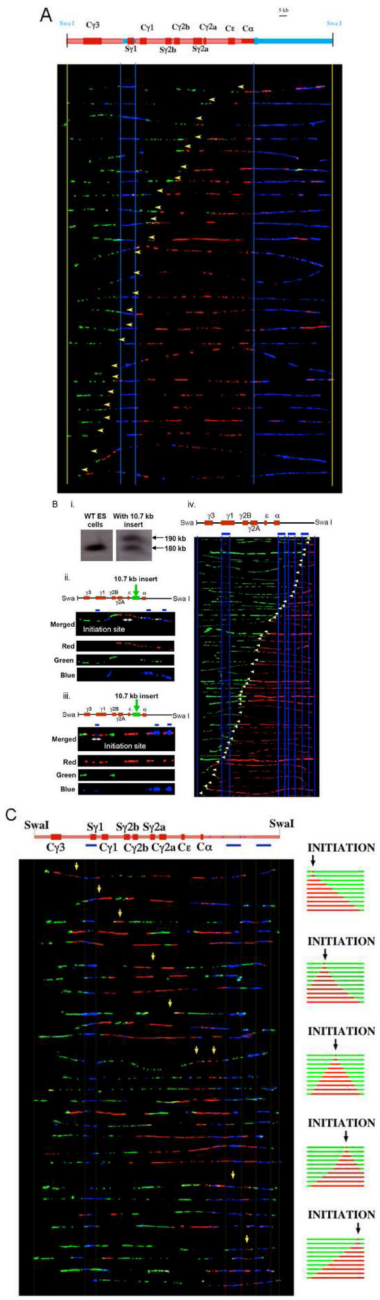

Figure 1. SMARD analysis of the mouse Igh locus

A. (Top) Map of the 180 kb SwaI segment containing the constant region genes of the mouse Igh locus. (Bottom) Replication forks progress predominantly in one direction through the constant region of the Igh locus in mouse ES cells. Cells were sequentially labeled with IdU and CldU to reveal DNA replication tracts. Chromosomal DNA was subsequently isolated and analyzed by SMARD (e.g., see [22]). Images of single DNA molecules show regions of IdU (red tracts) and CldU (green tracts) incorporation. The blue regions show the position of hybridization probes utilized to identify and align the SwaI DNA segments. The DNA molecules have been arranged such that positions of the red-to-green transitions (yellow arrowheads) occur from right to left. These transitions correspond to the location of the DNA replication fork at the completion of the IdU labeling period.

Initiation occurs downstream from these DNA molecules. **B.** In preB and proB cells, DNA replication initiates in the SwaI segment. These segments have internal origins indicated by the red patches surrounded by green. **C.** Insertion of a 10.7 kb initiation zone sequence into the SwaI element causes origin firing distal to the element. (Top) One SwaI molecule with DNA replication initiation sites was detected after the 10.7 kb sequence was inserted (green arrow). The merged image is shown above, with the individual channel images shown below. The earliest detected origin is indicated with a double white arrow. Two additional initiation events are also seen in the image. (Bottom) Another SwaI segment molecule with a replication initiation site observed outside of the 10.7 kb insertion.

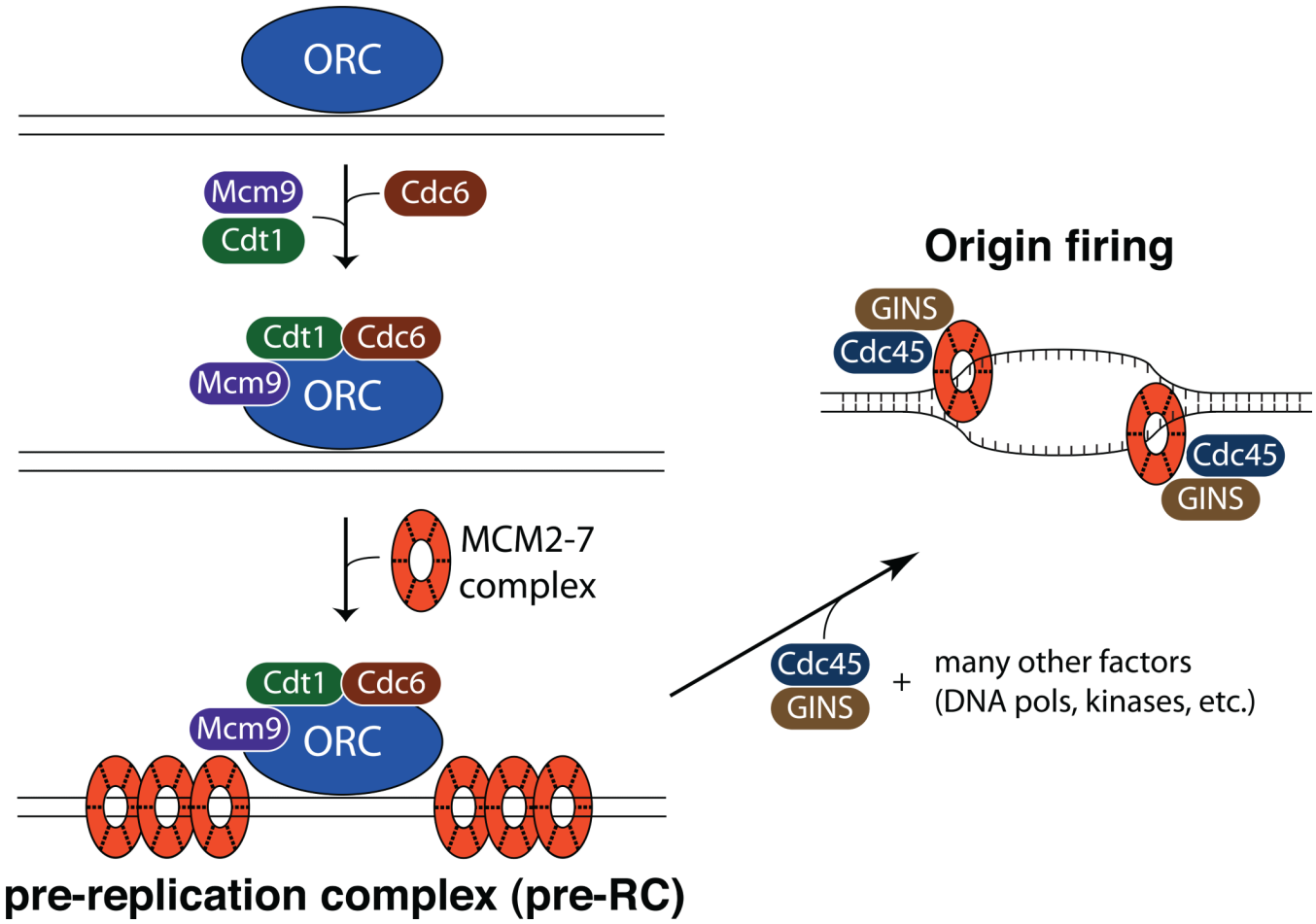

Figure 2. Schematic of pre-RC complex formation and origin firing in mammals

In mammalian cells during the G1 phase of the cell cycle, the ORC hexameric complex serves as an interactive platform for the sequential recruitment of licensing factors including Cdc6, Cdt1 and Mcm9. This leads to eventual loading of multiple copies of the MCM2-7 complex onto chromatin, generating the replication competent pre-RC. Activation of cyclin-dependent kinases (CDK) after passing the restriction point facilitates the association of additional factors with the pre-RC to form the pre-initiation complex (not shown). CDK and Dbf4-dependent kinase (DDK) activities eventually lead to release of the replicative helicase formed by MCM2-7, Cdc45, and GINS, and generation of two replication forks that migrate bidirectionally outward from the origin. To reduce complexity in this schematic, we do not show most of the essential replication factors, including DNA polymerases that would associate with the new replication forks.

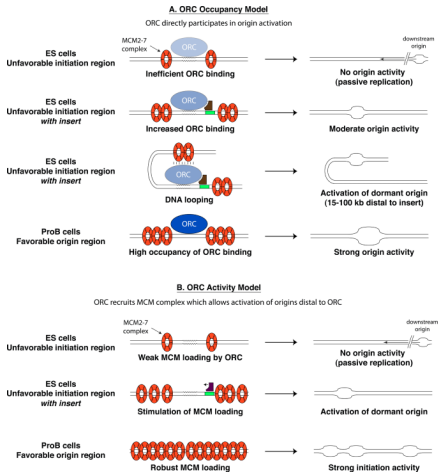

Figure 3. Models for differential origin activity

A. The first class of model postulates that a major determinant of origin activity is the occupancy of ORC binding. In the upper example, a weakly bound DNA element results in reduced ORC occupancy, as indicated by a transparent ORC (light blue oval). This reduced association would afford a correspondingly poorer licensing (less MCM loading) and little or no origin activity, leading to the DNA being replicated passively from a downstream origin (on the right). In the case of the 10.7 kb initiation region insertion into the murine Igh SwaI segment (second line), ORC occupancy could be increased by the ectopic DNA insert (green rectangle). In this example, the insert binds a factor (brown) that increases ORC binding (darker blue oval) and thereby causes weak but detectable origin activity (shown by a small replication bubble). Origin activity could occur outside of the insertion, because ORC binding may be increased in the general vicinity of the insert. Note that because of DNA looping, the bound ORC may have the potential to associate with MCM complexes bound to distal sequences, causing dormant origin activation at a distance (third line). The bottom example shows a DNA region that supports high occupancy of the ORC complex (dark blue oval) would also support efficient origin licensing (i.e., better MCM2-7 loading; red rings) and hence increased origin activity from that region (indicated by a large replication bubble). **B.** The second class of model proposes that the activity of ORC in loading the MCM complex, rather than the overall level of ORC binding, is the key feature that modulates origin activity. The top example indicates a DNA element that is unable to efficiently stimulate ORC activity, demonstrated by poorer loading of the MCM complex, and no origin activity. As in model A, the DNA would be replicated passively by a fork initiating from a downstream origin. Introduction of the ectopic element (middle example) provides a mechanism to stimulate ORC activity, leading to loading of additional MCM complexes and weak origin activity (a single small replication bubble). The bottom example depicts a DNA element that yields a highly-active ORC complex, indicated by the loading of multiple MCM2-7 complexes and efficient origin activity (signified by multiple replication bubbles along the DNA).