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SPOTLIGHT

# DNA replication licensing in stem cells: Gatekeeping the commitment to proliferation

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**Carroll et al. (2018. *J. Cell Biol.* https://doi.org/10.1083/jcb.201708023) developed a method to assess DNA replication licensing in tissues. They show that intestinal stem cells within wild-type crypts, but not in crypts with cancer-causing mutations, are largely unlicensed, suggesting that licensing may represent a rate-limiting step in the commitment to proliferation.**

Many tissues contain quiescent stem cells that can be induced to enter the cell cycle and generate progeny called transit-amplifying cells. These transit-amplifying cells proliferate and differentiate to replenish cells that are lost or die. An understanding of the mechanisms that control stem cells' decisions to enter the proliferative cell cycle would provide important insights into tissue homeostasis and regeneration. One limitation in our understanding of the activation of quiescent stem cells has been the lack of markers and landmarks for quiescent cells. Although the progression through S phase, G2, and mitosis involves changes that are easily visible, the transition in and out of quiescence is not associated with easily detectable changes to cells. With few markers or indications of whether a cell is deeply quiescent or about to divide, it has been difficult to develop clear models for stem cell activation. Carroll et al. provide a new methodology to determine whether cells in the G0/G1 phase of the cell cycle have passed through a critical step in the commitment to proliferation: the translocation of the MCM2-7 proteins from the cytoplasm to origins of DNA replication (Carroll et al., 2018). Applying this methodology to intestinal crypts allowed Carroll et al. (2018) to discover that whereas proliferating cells have chromatin-associated MCM2-7 proteins, stem cells mostly do not have MCM2-7 bound to chromatin.

The synthesis of large eukaryotic genomes initiates at origins of replication, specific, designated locations. At these origins, DNA is unwound and made accessible to DNA polymerases (Blow and Hodgson, 2002). Although some proteins remain continuously bound to these origins, the double-hexamer complex of the MCM2-7 proteins translocates from the cytoplasm to the chromatin during the previous mitosis through the beginning of G1 (Blow and Dutta, 2005). The MCM2-7 complex must be activated by cyclin-dependent kinases for DNA replication to initiate. Because origin licensing cannot occur after the middle of G1, the same portions of DNA are not replicated more than once (Truong and Wu, 2011). By extracting cytoplasmic but not chromatin-bound MCM2-7, Carroll et al. (2018) determined whether individual cells in a tissue contain licensed DNA (Blow and Hodgson, 2002). This allowed them to establish a milestone marker within the largely unmarked G0/G1 phase of the cell cycle. It also allowed them to test the hypothesis that quiescent cells can be defined as nondividing cells in an unlicensed state (Blow and Hodgson, 2002; Coller, 2007).

The intestinal epithelium is a tissue that undergoes continuous turnover to replenish lost cells. This replacement is largely performed by proliferating stem cells that differentiate into the epithelial cells that line the intestine. The most important source of these proliferative stem cells are stem cells that are mostly quiescent and divide only rarely when needed to replace the lost cells (Buczacki et al., 2013). The quiescent stem cells in the intestine mostly localize to the +4 position from the crypt base, are positive for stem cell marker Lgr5, and are negative for the secretory cell marker UAE. To better characterize the cell cycle in the different cell types within the crypt, Carroll et al. (2018) evaluated total Mcm2 levels as a marker for the Mcm2-7 complex in intestinal crypts. Mcm2 was expressed in $Lgr5^+$ cells, the main proliferative stem cells that divide to replace lost cells, and was absent in the fully differentiated, secretory Paneth cells. Mcm2 was also absent from the differentiated cells higher in the villi, consistent with a decline in the levels of Mcm2-7 proteins with differentiation (Eward et al., 2004).

The researchers then used their methodology to extract cytoplasmic Mcm2, while leaving Mcm2 bound to chromatin intact. They found that most Mcm2 had been removed, including from mitotic cells as expected, because Mcm2 is cytoplasmic during mitosis. Chromatin-bound Mcm2 was not present in differentiated cells distal to the transit-amplifying cells, even cells that

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were Ki-67 or EdU positive. The findings indicate that differentiated cells, even if they are passing through a final cell cycle, are no longer licensed. In the crypt base, >50% of the Lgr5$^+$ stem cells were unlicensed, whereas 30–40% of the Lgr5$^+$ cells were actively cycling, likely representing currently amplifying stem cells. Using flow cytometry, Carroll et al. (2018) found that most Lgr5$^+$ cells in crypts had low Mcm2-bound chromatin even if they had high Ki-67, indicating that licensing may be a more important determinant of cycling than other more commonly used markers. Their experimental data are most consistent with a model in which cells do not load MCM2-7 proteins until the cell has committed to cell cycle progression, at which time the protein complex is rapidly loaded. Carroll et al. (2018) further distinguished among stem cells based on the recency of division. They activated expression of fluorescently labeled histone proteins and then turned off expression of these genes. Cells that remained fluorescent 7 d later were cells that had not divided during the intervening period and represented the most deeply quiescent cells in the crypt. These label-retaining cells had low levels of total Mcm2 proteins. These deeply quiescent cells are thus dissimilar from the "active" intestinal stem cells. The label-retaining cells had low levels of total Mcm2 proteins, whereas the active stem cells had high levels of Mcm2 proteins that were not bound to chromatin; the active stem cells were thus poised but unlicensed.

To better understand the role of signaling pathways in the establishment of licensed and unlicensed states, Carroll et al. (2018) generated intestine-derived organoids. These organoids had a higher fraction of cells with DNA-bound Mcm2 than was found in intestines, indicating that the stem cell niche in vivo may contribute to reducing the rate of licensing. Short-term treatment of organoids with an inhibitor of EGF signaling resulted in cells accumulating in an unlicensed G1 state with high Mcm2 and Ki-67 but low chromatin-bound Mcm2. After 4 d of EGF inhibition, cells maintained high total Mcm2, but there was a shift so that more cells had low Ki-67 expression. These findings suggest that EGF-based signaling may be important for the licensing of Mcm2.

Inspired by previous literature reporting that established cell lines lose the unlicensed G1 state and immediately license their origins upon exit from mitosis (Friedrich et al., 2005), Carroll et al. (2018) investigated the effects of loss of adenomatous polyposis coli (APC), a protein that promotes degradation of β-catenin in the Wnt pathway (Stamos and Weis, 2013). APC loss is a frequent early event in colorectal cancer (Kwong and Dove, 2009). Comparing organoids from WT mice and mice with mutations in the *APC* gene revealed that APC inactivation resulted in significantly fewer unlicensed cells and more cells in the early phase of licensing. Carroll et al. (2018) sought to understand whether DNA licensing might be associated with the restriction point. The restriction point, defined as the point in the cell cycle after which a cell is irreversibly committed to divide, is thought to be enforced by phosphorylation of the retinoblastoma (Rb) protein. To determine whether the change in the fraction of cells with licensed origins with APC inactivation correlates with a change in the restriction point, Carroll et al. (2018) monitored the phosphorylation status of Rb. They found that although Rb proteins in cells in WT organoids were mostly hypophosphorylated, there were significantly more hyperphosphorylated Rb proteins in cells in *APC* mutant organoids. The findings are consistent with a connection between the Rb-based restriction point and origin licensing.

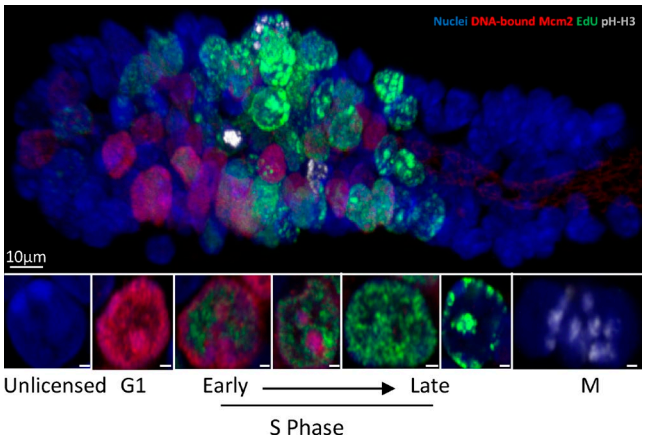


Figure 1. **An intestinal crypt is shown with markers for cell cycle phases.** The base of the crypt is on the left of the image. G0, early G1, and G2 cells are indicated with a blue dye for nuclei. The methodology used by Carroll et al. (2018) to monitor chromatin-bound Mcm2 identifies cells in early G1 and is indicated in red. Cycling cells are positive for EdU and are indicated in green. Phospho–histone H3, a marker of M phase, is visualized as whitish dots. The majority of cells in the transit-amplifying compartment were positive for EdU, indicating that they were actively cycling. At the base of the crypt, most of the cells are unlicensed and are likely in G0. There are some cells with chromatin-bound Mcm2 between the crypt base and the zone of actively proliferating EdU-positive cells. Image republished from Carroll et al. (2018) Fig. 3 A.

Collectively, Carroll et al. (2018) developed a method that allows them to monitor DNA licensing in intact crypts and organoids. Their data reveal that there is a common state for stem cells of the intestine in which Mcm2 is expressed but has not translocated to chromatin. These cells can have high Ki-67, indicating that they have some of the markers of proliferative cells but are held in check by lack of licensing (Fig. 1). The findings by Carroll et al. (2018) raise the possibility that loading of MCM2-7 proteins to chromatin represents an important gatekeeping event for the transition between quiescence and proliferation. The findings also suggest that the longer G0/G1 in stem cells reflects an extended unlicensed period. These findings are supported by the loss of the unlicensed state in cells with cancer-causing nonsense mutations in *APC*.

Carroll et al. (2018) raise many interesting questions for further study. What is regulating the translocation of Mcm2-7 proteins to chromatin? How do Mcm2 protein levels correlate with the levels of the E2F proteins? Are there unlicensed periods in the cell cycles of stem cells in other tissues, such as tissues that are less highly proliferative? Is there a role for DNA licensing in controlling the replication of other types of cells or only stem cells? Do all oncogenes, like APC, affect licensing and cause cells to remain continuously licensed? Is origin licensing important for other hyperproliferative cell states? The deeply G0 cells lost Mcm2 completely. Are there any landmarks that could be used to better classify deep G0?

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