When cells are stimulated by growth factors, they make a critical choice in early G1 phase: proceed forward to S phase, remain in G1, or revert to G0 phase. Once the critical decision is made, cells execute a fixed program independently of extracellular signals. The specific stage at which the critical decision is made is called the restriction point or R-point. The existence of the R-point raises a major question: what is the nature of the molecular machinery that decides whether or not a cell in G1 will continue to advance through the cell cycle or exit from the cell cycle? The R-point program is perturbed in nearly all cancer cells. Therefore, exploring the nature of the R-point decision-making machinery will provide insight into how cells consult extracellular signals and intracellular status to make an appropriate R-point decision, as well into the development of cancers. Recent studies have shown that expression of a number of immediate early genes is associated with the R-point decision, and that the decision-making program constitutes an oncogene surveillance mechanism. In this review, we briefly summarize recent findings regarding the mechanisms underlying the context-dependent R-point decision.

Keywords: BRD, PcG complex, restriction point, RUNX, TrxG complex

INTRODUCTION

Cells consult their extracellular environment, and the growth-regulating signals within it, during a discrete window of time in G1 phase of the cell cycle. Once cells have moved through this window, they proceed through S, G2, and M phases. However, if growth factors are removed before a specific time point, cells will fail to proceed toward S phase, and instead remain in G1 or revert to G0 phase (Pardee, 1974). This total dependence on extracellular signals, followed by entrance in late G1 into a state of relative independence, indicates that an important decision must be made before the end of G1. Precisely at this point, a cell must ‘make up its mind’ whether it will remain in G1, retreat from the active cycle into G0, or advance into late G1 and S phase. This critical decision is made at the restriction point or R-point (Malumbres and Barbacid, 2001; Pardee, 1974; Weinberg, 2014).

Time-lapse microscopy of asynchronously cycling Swiss 3T3 cells showed that cycling cells are sensitive to serum withdrawal for only the first 3 to 4 h after mitosis, implying that the R-point is within this interval (Zetterberg and Larsson, 1985). Subsequent studies revealed that the R-point occurs 3 to 4 h after mitogenic stimulation in most mammalian cells (Zetterberg et al., 1995). The concept of the R-point heralded the subsequent discovery of the cell-cycle engine (Nurse, 2000; Pardee, 1974; Sherr and Roberts, 1999). In addition, the R-point is deregulated in most cancer cells (Pardee, 1974; Weinberg, 2014). Therefore, understanding how cells make a fate decision at the R-point should provide insight into how the cell cycle is controlled and how tumors develop (Blagosklonny, 2006).
The DNA-binding transcription factor RUNX3, which plays pivotal roles in lineage determination, defends against oncogenic K-RAS–induced tumorigenesis (Ito et al., 2015). Deletion of Runx3 in mouse lung results in development of lung adenomas and accelerates oncogenic K-RAS–induced progression into adenocarcinomas (Lee et al., 2013). In mouse embryonic fibroblasts, Runx3 deletion perturbs the R-point, leading to transformation (Chi et al., 2017). Recent work showed that RUNX3 functions as a pioneer factor that plays key roles in R-point–associated induction of immediate early genes, including p21\(^{\text{WAF1/Cip1}}\) (hereafter p21) and p14\(^{\text{ARF}}\) (p14\(^{\text{ARF}}\) in human, hereafter ARF). In this review, we summarize how RUNX3 contributes to the R-point decision in collaboration with histone modifiers, chromatin-remodeling complexes, the basal transcriptional machinery, and Polycomb group (PcG) proteins.

**CHROMATIN DYNAMICS ASSOCIATED WITH R-POINT REGULATION**

When extracellular mitogenic signaling is maintained up to the R-point, transcription of R-point–associated target genes is activated (Chi et al., 2017). For a silent gene to be induced, the chromatin structure of its chromosomal locus must be opened. Chromatin structures and functions are modulated by covalent modification of specific residues within the amino-terminal tails of histones; the unique combination of modifications has been described as the histone code (Mills, 2010). Trithorax group (TrxG) proteins establish histone modifications that activate transcription, whereas PcG proteins establish histone modifications that repress transcription. TrxG proteins consist of two categories: histone modifiers (Strahl and Allis, 2000) and nucleosome remodelers (Vignali et al., 2000). TrxG histone modifiers include the mixed-lineage leukemia (MLL) protein, which methylates histone H3 at lysine 4 (H3K4-me), a histone mark that favors transcriptional activation. Nucleosome remodelers of TrxG complex contain SWI/SNF complex, which facilitate the binding of transcription factors and basal transcription machinery (Imbalzano et al., 1994). PcG complexes include two categories: Polycomb repressor complexes 1 and 2 (PRC1 and PRC2). The PRC2 complex contains Enhancer of Zeste Homolog 2 (EZH2), which trimethylates histone H3 at lysine 27 (H3K27-me), a characteristic of inactive chromatin (Cao et al., 2002).

Gain of PcG and loss of TrxG is a common theme in human cancer, indicating that PcG and TrxG are involved in regulation of tumor suppressors: PcG suppresses and TrxG activates tumor suppressors. ARF, which induces cell-cycle arrest and apoptosis by facilitating p53 activity in response to aberrant oncogene activation (Efeyan and Serrano, 2007; Kamijo et al., 1997; Palmero et al., 1998), is regulated by PcG and TrxG. During normal proliferation, PcG complexes (PRC1 and PRC2) and histone deacetylases (HDACs) bind the ARF locus, thereby inhibiting senescence. In response to oncogenic RAS, TrxG-mediated chromatin dynamics override PcG-mediated repression, thereby inducing ARF/p53-mediated senescence (Mills, 2010).

**ENTERING INTO THE R-POINT AND ACTIVATION OF R-POINT-ASSOCIATED GENES**

Soon after mitogenic stimulation (within 1 h after stimulation), histone H4 and RUNX are acetylated by p300 acetyltransferase. BRD2 contains two bromodomains (BD1 and BD2), and each bromodomain interacts with a distinct protein: BD1 interacts with acetylated RUNX3, whereas BD2 interacts with acetylated histone H4. The p300–RUNX3–BRD2–histone complex is formed 1 to 2 h after serum stimulation, and binds to the promoters of the genes encoding p21 and ARF (Lee et al., 2019a). The complex dissociates 4 h later (Lee et al., 2019a). These observations suggest that a large complex containing RUNX3, BRD2, p300, and histone forms at the promoters of p21 and ARF at the R-point: within this complex, BRD2 grips both RUNX3 and histone through its two BDs. A schematic diagram of the complex formed at target loci soon after serum stimulation is shown in Figure 1.

BRD2 participates in multiprotein transcription complexes such as Mediator, recruits the SWI/SNF chromatin-remodeling complex (Denis et al., 2006), and allows RNA polymerase II to transcribe through the nucleosome (LeRoy et al., 2008). Interestingly, TAF1 and TBP form a complex with RUNX3 and BRD2 1 h after serum stimulation (Fig. 1). Similarly, MLL1/5 (activating histone modifiers) and the components of the SWI/SNF complex, Brg-1 and BAF-155, interact with RUNX3 and BRD2 simultaneously 1 to 2 h after serum stimulation, and subsequently dissociate (Fig. 1). These results demonstrate that the p300–RUNX3–BRD2–histone complex interacts with MLL1/5 as well as the SWI/SNF and TFII D complexes before the R-point.

ARF is a target of RUNX3 (Lee et al., 2013) that is critical for the life and death of cells; thus regulation of its expression could represent the R-point decision. Lee et al. (2019a) showed that MLL1/5, SWI/SNF, and TFII D are recruited to the ARF promoter locus, but not when the RUNX3 binding site is deleted. Consistent with this, MLL1/5, SWI/SNF, and TFII D are not recruited to the ARF promoter in H460 cells, which do not express RUNX3 but are recruited after ectopic expression of RUNX3. These results demonstrated that the MLL1/5, SWI/SNF, and TFII D complexes are recruited to the ARF promoter, and that their recruitment is guided by RUNX3. The large RUNX3-containing complex formed before the R-point has been designated as the R-point–associated RUNX3-containing activator complex (Rpa-RX3-AC) (Fig. 1).

**RUNX3 IS A PIONEER FACTOR OF THE R-POINT**

In HEK293 cells, H3K27-me3 (a repressive histone modification) is highly enriched in the ARF promoter region prior to serum stimulation. Notably, H3K27-me3 is replaced by H3K4-me3 (an activating histone modification) 1 to 4 h after serum stimulation, whereas H3K27-me3 is recruited to region 8 h later. Similarly, H4K12-ac (an activating histone modification) is detected in the ARF promoter region 1 to 4 h after stimulation and is subsequently erased. Consistent with this, induction of ARF leading to p53 stabilization occurs 1 to 2 h after stimulation (Lee et al., 2019a). By contrast, in HEK293 cells in which the RUNX binding site was deleted from the ARF pro-
Fig. 1. The RUNX3-BRD2-nucleosome complex recruits SWI/SNF and TFIID. Schematic illustration of sequential molecular events at RUNX3 target loci during R-point regulation. RUNX3 binds to condensed chromatin marked by H3K27-me3 (inhibitory mark). p300 recruited to the loci acetylates RUNX3 and histones. Then, BRD2 binds both acetylated RUNX3 and acetylated histone through its two bromodomains. Soon after, SWI/SNF, MLL1/5 and TFIID are recruited to the loci through the C-terminal region of BRD2 to form Rpa-RX3-AC. H3K27-me3 is replaced by H3K4-me3 (activating mark) by MLL1/5.

moter, the H3K27-me3 modification was maintained, and H4K12 was not acetylated for a long time after serum stimulation (Lee et al., 2019a). These results demonstrate that RUNX3 interacts with its binding site and opens and activates the chromatin structure of target loci 1 to 2 h after serum stimulation, and then subsequently closes and inactivates the loci 8 h later.

For induction of a silent gene, a special DNA-binding transcription factor must access the inactive chromatin and locally open it to make it competent for binding of other factors. These special transcription factors, which initiate regulatory events, are known as "pioneer transcription factors" (Zaret and Carroll, 2011). Because the R-point-associated activation of the ARF locus is dependent on RUNX3 protein and the presence of a RUNX binding site in the ARF promoter, RUNX3 is considered as a pioneer factor of the R-point (Fig. 1).

ROLES OF p21 AND CYCLIN D–CDK4/6 IN TRANSITION OF R-POINT COMPLEXES

Lee et al. (2019a) showed that BRD2 interacts with RNF2 and BMI1 (components of PRC1) 4 to 8 h after mitogenic stimulation. At that time, RUNX3 associates with Cyclin D1 and HDAC4, but dissociates from BRD2. Also, EED and EZH2 (components of PRC2) interact with RUNX3 8 h after stimulation. Consistent with this, ChIP analysis revealed that Cyclin D1, HDAC4, and EZH2 are recruited to target chromatin loci after the R-point, at a time when H4K12-ac is absent and H3K27-me3 is enriched at the target chromatin loci. After 4 h, RNF2 briefly interacts with RUNX3 and BRD2, and binds to target chromatin loci, suggesting that PRC1 and Rpa-RX3-AC form a transient complex immediately before the Rpa-RX3-AC is destroyed (about 3 h after mitogenic stimulation). The transient construction has been designated as the R-point-associated RUNX3-containing transient complex (Rpa-RX3-TR) (Fig. 2).

At 4 to 8 h after mitogenic stimulation, RUNX3 and BRD2 are present in separate complexes: RUNX3 is in complex with Cyclin D1, HDAC4, and PRC2, which remain bound to target chromatin loci, whereas BRD2 forms the BRD2–PRC1 complex, which was released from the loci. Because the RUNX3–Cyclin D1–HDAC4–PRC2 complex closes and inactivates target chromatin loci, it has been designated as the R-point-associated RUNX3-containing repressor complex (Rpa-RX3-RE) (Fig. 2).

The R-point transition is governed by R-point-associated proteins (R-proteins), which include cyclins, cyclin-dependent kinase (CDKs), p21, p27, E2F, and pRB, with pRB serving as the primary molecular regulator (Weinberg, 2014). The complexes of CDKs and their cyclin partners are responsible for sending out signals from the cell-cycle clock to a number of responder molecules that carry out the actual work of moving the cell through its growth and division cycle. During much of the G1 phase of the cell cycle, CDK4 and CDK6 (CDK4/6) are guided by and depend upon their association with D-type cyclins (D1, D2, and D3). After the R-point in late G1, the E-type cyclins (E1 and E2) associate with CDK2 to enable the phosphorylation of appropriate substrates required for entry into S phase, making the R-point decision irreversible.

In the case of Cyclin D1, which is the best studied of the three D-type Cyclins, growth factor-activated signal cascades result in rapid accumulation of Cyclin D1. Conversely, removal of growth factors from the growth medium results in an equally rapid collapse of the Cyclin D1 level. Therefore, D-type Cyclins must serve as regulators of the R-point, conveying signals from the extracellular environment to the cell-cycle clock operating in the nucleus. In early and mid-G1 phase, Cyclin D–CDK4/6 phosphorylates pRB prior to passing through the R-point gate, and Cyclin E–CDK2 further phosphorylates pRB and drives the cell cycle into S phase. Therefore, the R-point decision must be made after formation of the Cyclin D–CDK4/6 complex and before formation of the cyclin E–CDK2 complex. In other words, during the R-point decision, which requires substantial time for collection of extracellular signals and intracellular information, the Cyclin D–CDK4/6 complex must be formed, whereas the Cyclin E–CDK2 complex must not be formed. In that case, how is the timing of formation of these two complexes regulated in an R-point-dependent
One hour after mitogenic stimulation, BRDs bind both acetylated RUNX1 (M), which share a highly conserved Ub, ubiquitinates histones. The cell cycle then progresses toward S-phase. If K-RAS is constitutively activated, RAS-MEK signal is not downregulated, and Rpa-RX-AC is maintained for a long time; consequently, the cell cycle arrests or the cell undergoes apoptosis or senescence.

**MEMBERS OF THE RUNX FAMILY**

In mammals, there are three RUNX family genes *(RUNX1, RUNX2, and RUNX3)*, which share a highly conserved...
Runt-domain. All three RUNX family genes show distinct tissue-specific expression patterns and are intimately involved in carcinogenesis (Ito et al., 2008; 2015). In addition, not only BRD2, but also other BRD family members (BRD3, BRD4, and BRDT) contain two distinct, highly conserved bromodomains that recognize acetylated lysine residues (Filippakopoulos et al., 2012). BRD family members are also mutated in various tumors (Belkina and Denis, 2012).

Recently, Lee et al. (2019b) showed that all RUNX family members form complexes not only with BRD2, but also with BRD3 and BRD4 2 h after serum stimulation, and that these complexes are subsequently destroyed. Importantly, when the constitutively active form of K-RAS (K-RAS<sup>G12V</sup>) is expressed, all complexes were maintained for a long time, not only in the absence of serum stimulation, but also after serum stimulation. These results indicate that the RUNX and BRD families are involved in the lineage-specific R-point decision-making machinery, and thus contribute to oncogene surveillance (Fig. 2).

### PROSPECTS

Considering the fundamental role of the R-point decision-making machinery during normal cell differentiation and proliferation, the decision-making machinery must be able to recognize the status and environment of the cell, enabling the cell to make an appropriate decision. Recent studies revealed that concerted action of RUNX family members, chromatin remodelers, histone modifiers, the basal transcriptional complex, Polycomb complexes, and cell-cycle regulators contribute to the R-point decision, in which RUNX proteins function as pioneer factors. These recently identified R-point decision-making processes provide important clues about fundamental questions regarding how chromatin is selectively opened and closed in a signal-dependent manner, as well as how cells recognize and defend against oncogenic RAS signals. Therefore, identification of RUNX family members as pioneer factors of the R-point and subsequent molecular events represents a significant advance in cancer biology, and could potentially provide a new strategy for the development of anti-cancer drugs.

### Disclosure

The authors have no potential conflicts of interest to disclose.

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