



Lincoln's Inn Fields

Molecular Oncology

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Our research focuses on the regulation of the *INK4b-ARF-INK4a* tumour suppressor locus and its role in the implementation of cellular senescence, the state of permanent growth arrest elicited by various forms of stress. As well as serving as a front line defence against oncogenic insults, senescence sets limits on the proliferative potential of stem and progenitor cell populations. The current priority is to determine how oncogenic signalling affects transcription of the locus by altering the state of histone modifications in the neighbouring chromatin.

The *INK4b-ARF-INK4a* locus encodes three proteins, each of which is capable of bringing cell proliferation to a halt. The two INK4 proteins, p16^{INK4a} and p15^{INK4b}, do so by blocking the phosphorylation of the retinoblastoma protein (pRb) by cyclin dependent kinases, whereas p14^{ARF}, so named because it exploits the second exon of *INK4a* in an alternative translational reading frame, blocks the ubiquitin-mediated turnover of p53. The locus is kept transcriptionally silent in most normal cells *in vivo* but is activated by a variety of intracellular and extracellular cues, such as prolonged mitogenic stimulation, telomere erosion, oxidative stress or the presence of an activated oncogene. Explanting cells into tissue culture is enough to activate the locus, leading

eventually to senescence. Whereas p16^{INK4a} seems to be the major determinant of senescence in human cells, p15^{INK4b} assumes this role in chicken cells, presumably because chickens have lost the capacity to encode p16^{INK4a}. In contrast, senescence in mouse cells is primarily dependent on the ARF/p53 pathway.

Regulation of *INK4b-ARF-INK4a* by Polycomb group proteins

Current evidence suggests that in stem and early progenitor cells, the *INK4b-ARF-INK4a* locus is transcriptionally repressed by the actions of the Polycomb group (PcG) of proteins. These proteins participate in at least two types of multi-component complex, termed Polycomb repressive complexes 1 and 2 (PRC1 and PRC2). PRC2 contains the histone methyltransferase EZH2 which catalyses trimethylation of histone H3 on lysine 27 (H3K27me3). This mark is recognised by the PRC1 complex which mono-ubiquitylates histone H2A on lysine 119, thereby shutting down transcription (Figure 1). Whereas the PRC1 complex in *Drosophila* comprises stoichiometric amounts of four proteins, Pc, Psc, Ph and Sce, each has multiple orthologues in mammalian cells.

To try to define the PRC1 complexes that regulate *INK4a*, we have used tandem affinity purification and mass spectrometry to identify proteins that co-purify with the Pc homologue CBX7, and the Psc homologues BMI1 and MEL18. Our analyses suggest that although there are multiple variants of PRC1 in human cells, each contains a single representative of the Pc, Psc, Ph and Sce families. Surprisingly, we find that multiple PRC1 components are associated with chromatin at the *INK4a* locus and that shRNA-mediated knockdown of any one of these can result in loss of H3K27me3 at the locus and derepression of *INK4a*. The most likely explanation, for which we are seeking additional evidence, is that *INK4a* is regulated by a complex of PRC1 complexes.

Proteins that co-purify with Polycomb complexes

As well as known PcG proteins, the mass spectrometry identified non-PcG components that may be implicated in PRC1 function. These include two ubiquitin proteases that might be involved in editing the mono-ubiquitylation of histone H2A by PRC1 or possibly in removing ubiquitin from H2B. This would serve to reinforce transcriptional repression by the complex. We also found an RNA helicase and are exploring the attractive idea that RNA interference or non-coding RNAs are required to establish PcG-mediated gene silencing. Importantly, knockdown of these non-PcG proteins with shRNA results in loss of PRC1 binding at the *INK4a* locus and p16^{INK4a} dependent senescence. We are using similar strategies to investigate protein kinases that consistently co-purify with PRC1 complexes, and in particular asking whether they phosphorylate histones or components of PRC1.

Reversal of PcG-mediated repression by oncogenic signalling

In collaboration with colleagues at the MRC Clinical Sciences Centre in London and Mount Sinai Medical Center in New York, we have been investigating how signalling from the RAS-RAF-MEK pathway impacts on PcG-mediated regulation of the *INK4b-ARF-INK4a* locus. Oncogenic RAS causes a reduction in H3K27me3 at the *INK4a* promoter by down-regulating the EZH2 methyl transferase and upregulating one of the recently described H3K27me3 demethylases, JMJD3. In human fibroblasts, JMJD3 activates *INK4a*, but not *ARF*, and causes p16^{INK4a}-dependent arrest. In contrast, in mouse embryo fibroblasts, JMJD3 activates both *Ink4a* and *Arf* and

elicits a p53-dependent arrest, echoing the effects of RAS in this system. Our findings directly implicate JMJD3 in the regulation of *INK4a/ARF* during oncogene-induced senescence and suggest that JMJD3 has the capacity to act as a tumour suppressor.

Interestingly, the effects of JMJD3 do not extend to *INK4b* despite clear evidence that p15^{INK4b} is also induced by RAS. In contrast, the MYC oncogene appears to have opposing effects on *INK4b* and *INK4a*. We are therefore conducting more detailed analyses of histone modifications (e.g. H3K27, H3K36, H3K4 etc) throughout the *INK4b-ARF-INK4a* locus and how they are altered in response to various agents and stresses. We are also extending these studies to chicken cells in which the PRC1 binding region of *INK4a* is missing.

Reciprocal regulation of INK4a and INK4c

In studying the role of the INK4 family in senescence, we noticed that when p16^{INK4a} accumulates during replicative or oncogene induced senescence, the expression of p18^{INK4c} is turned off. This occurs at the level of transcription and is not simply a case of one protein competing with the other for binding to CDKs. Curiously, much of the p18^{INK4c} in proliferating fibroblasts is specifically associated with CDK6. As oncogenic RAS has reciprocal effects on p16^{INK4a} and p18^{INK4c}, we are extending the analyses of histone modifications to include *INK4c*.

Publications listed on page 125

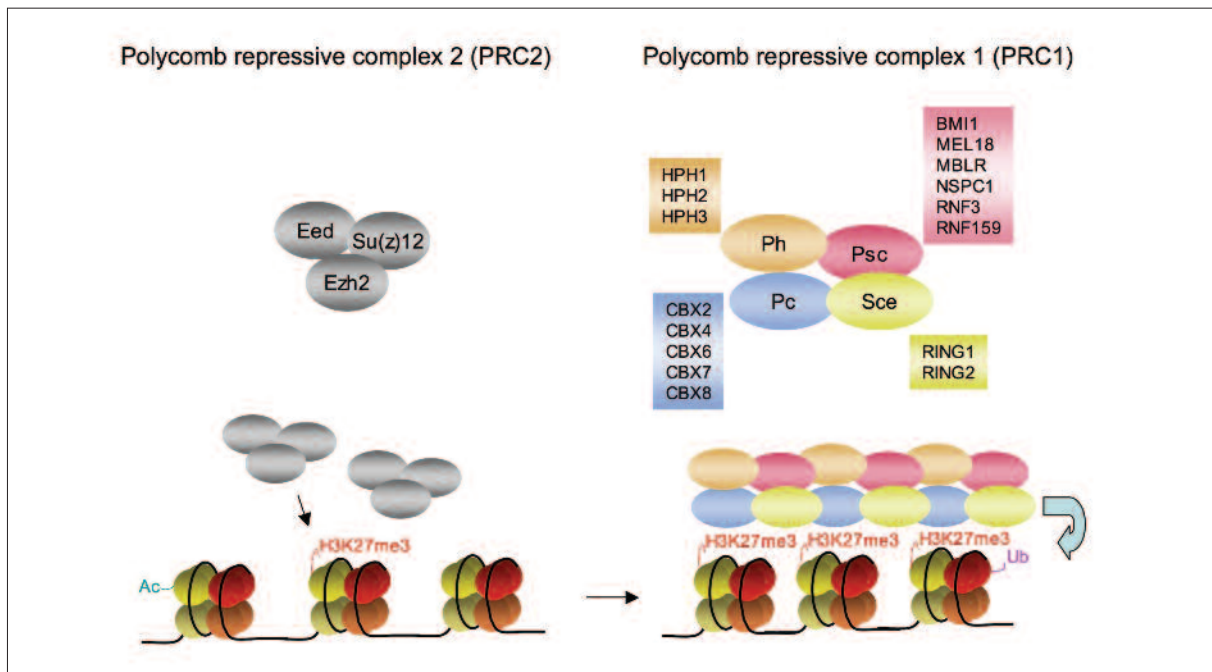


Figure 1. Transcriptional repression by the Polycomb group proteins. The PRC2 complex, comprising Ezh2, Eed and Su(z)12, establishes epigenetic marks in chromatin by tri-methylating histone H3 on lysine 27 (H3K27). This mark is recognised by the PRC1 complex, which mono-ubiquitylates histone H2A on lysine 119, leading to transcriptional repression. Whereas the PRC1 complex in *Drosophila* comprises stoichiometric amounts of Pc, Psc, Ph and Sce, there are multiple orthologues of these proteins in mammalian cells, as indicated in the coloured boxes.