## PLENARY SESSION IV
## OTHER MOLECULAR TARGETS IN APL AND BEYOND

### MURINE MODELS OF APL INDUCED BY CO-EXPRESSION OF THE PML-RARα FUSION WITH EITHER FLT3-ITD OR ONCOGENIC K-RAS ALLELES

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The expression of fusion proteins in hematopoietic progenitor cells containing the retinoic acid receptor alpha (RAR), including PML-RARα, PLZF-RARα and others, is associated with a phenotype of acute promyelocytic leukemia (APL). However, several lines of evidence indicate that expression of these alleles alone is not sufficient to induce the APL phenotype. These include the observation that ~30% of patients with APL have activating mutations in FLT3, and ~5-10% have activating mutations in N-RAS or K-RAS. In addition, in murine models of disease, expression of PML-RARα directed to the promyelocyte compartment using transgenesis with a Cathepsin G promoter results in a development with long latency and incomplete penetrance, indicating that additional mutations are required for pathogenesis of APL in addition to the PML-RARα fusion. Although penetrance can be enhanced by expression of the reciprocal RARα-PML fusion, or by expression of PML-RARα from the endogenous Cathepsin G promoter using knock-in strategies, these models retain a long latency and often have associated non-random cytogenetic abnormalities indicating a requirement for additional mutation(s).

We have developed two murine models of disease in which a FLT3-ITD allele is co-expressed with the PML-RARα fusion using retroviral transduction, or in which oncogenic K-RAS is co-expressed from its endogenous promoter under conditional control in a Cathepsin G transgenic model. These models of cooperativity thus recapitulate known genotypic abnormalities associated with the human disease. We observe that co-expression of either FLT3-ITD or oncogenic K-RAS results in a short latency and fully penetrant APL-like phenotype.

Further, hematopoietic progenitors expressing both alleles demonstrate properties of self-renewal, including serial transplantability and serial replating activity in methylcellulose. These models provide useful platforms for testing therapies that specifically target the FLT3-ITD, oncogenic K-RAS, or PML-RARα leukemia oncogenes.

### ROLE OF THE SUMO PATHWAY IN CELLULAR SENESCENCE AND DEVELOPMENT

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Covalent modification by SUMO regulates a wide range of cellular processes including transcription, cell cycle and chromatin dynamics. Recently, sumoylation was shown to play an important role in APL pathogenesis as a sumoylation-deficient PML-RAR oncoprotein fails to induce acute leukemia in a mouse APL model. To address the biological function of the SUMO pathway in mammals, we generated mice deficient for the SUMO E2-conjugating enzyme Ubc9. Ubc9–deficient embryos die at the early post-implantation stage. In culture, Ubc9 mutant blastocysts are viable but fail to expand after 2 days showing apoptosis of the inner cell mass. Loss of Ubc9 leads to major chromosome condensation and segregation defects. Ubc9-deficient cells also show severe defects in nuclear organization including nuclear envelope dysmorphy and disruption of nucleoli and PML Nuclear Bodies. Moreover, RanGAP1 fails to accumulate at the nuclear pore complex in mutant cells that show a collapse in Ran distribution. Together, these findings reveal a major role for Ubc9, and, by implication, for the SUMO pathway, in nuclear architecture and function, chromosome segregation and for embryonic viability in mammals.

In a parallel study performed in human primary fibroblasts, we could identify the E3 SUMO ligase PIASy as a novel regulator of cellular senescence and apoptosis. PIASy induces senescence by activating the tumour suppressor proteins p53 and Rb. This activation entails PIASy-mediated sumoylation of p53 and Rb-dependent co-repression of E2F-response genes in senescence-associated heterochromatin foci. In the absence of Rb, PIASy expression leads to E2F/p53-dependent apoptosis. The anti-proliferative function of PIASy requires its E3 SUMO ligase activity and is specific for this member of the PIAS family of E3 SUMO ligases. Inhibition of PIASy E3 ligase function by E6 prevents induction of senescence. These data provide the first evidence for a direct role of an E3 SUMO ligase, and by implication of the SUMO pathway, in cellular senescence, apoptosis and tumour suppression.

## NEW INSIGHTS INTO THE TRANSCRIPTION REPRESSION BY PML-RARα : INVOLVEMENT OF N-COR / SMRT-TBLR1-HDAC3 COMPLEX

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*Introduction*

Transcription repression by PML-RARα chimeric transcription factor plays an important role for the leukemogenesis in APL. More than 10 years, all-*trans* retinoic acid (ATRA) has been administrated for APL patients, and a dramatic improvement of cure rate of APL has been achieved. Recently, it becomes widely recognized that ATRA is one of an excellent molecular targeting reagent for APL.[4, 5, 7, 8, 13] ATRA works directly on PML-RARα protein *in vivo*, and the aberrant transcription repression on many target genes can be released by administration of ATRA at the pharmacological concentration. Transcription de-repression on PML-RARα target genes are thought to be a key mechanism of the molecular targeting therapy and *transcription therapy* in APL.

*N-CoR/SMRT complex as a transcription co-repressor of PML-RARα*

We are currently interested in detailed mechanisms of transcription repression by PML-RARα *in vivo*. N-CoR (Nuclear receptor co-repressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptors) are known to be key molecules for the transcription repression of nuclear receptors including retinoic acid receptor (RAR) and thyroid hormone receptor (TR).[1, 9, 11]

Previously, it has been reported that N-CoR/SMRT forms large protein complexes that contain relatively smaller-molecular-sized functional proteins including transcription co-repressors Sin3 and HDAC1 (histone deacetylase 1).[2, 4, 8, 13, 14] Recent 5 years, several kinds of N-CoR/SMRT complexes other than Sin3/HDAC1 complex has been reported.[10]

Among them, N-CoR/SMRT-TBL1/R1 (transducin beta like protein 1/relating protein) complex is relatively well characterized by several laboratories.[6,12,17,19]

Furthermore, N-CoR/SMRT complexes containing class II HDAC proteins (i.e. HDAC)[4, 5, 7, 9,10] has been also reported.[3] N-CoR/SMRT-TBL1/R1 complex is firstly purified in three different laboratories using the biochemical strategies, and several transcription regulator proteins are identified in the complex. TBL1/R1, HDAC3, Kaiso,[17] and GPS2[19] (G-protein pathway suppressor 2) are the major components of this large protein complex. TBL1/R1 have WD40 repeat which is known to be a histone-binding motif. Recently, TBL1/R1 binding with deacetylated histone is demonstrated, and is required for the transcription repression of unliganded nuclear hormone receptors.[15,16,18]

HDAC3 is one of class I histone deacetylases just like HDAC1. Histone deacetylation of specific chromatin locus by HDACs is closely related to transcription repression of target genes. Kaiso is a methyl CpG binding protein belonging to the BTB/POZ family of transcription factors. GPS2 is a protein involved in intracellular signaling. Some of them work as transcription repressor, and they may be works in a coordinated manner on specific promoters. These co-factors do not interact with transcription factors directly. N-CoR and SMRT is recruited to transcription factors on specific gene promoters, and these co-factors, such as TBL1/R1 and HDAC, work for the transcription repression of specific gene expression. Transcription factors that interact with N-CoR/SMRT-TBL1/R1 complex are indicated in Figure 1.

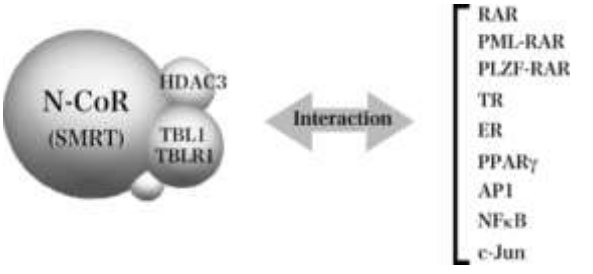


**Figure 1.**

*N-CoR/SMRT-TBLR1 complex in transcription regulation by PML-RARα*

Previously, we have shown that unliganded PML-RARα interacts with N-CoR/SMRT-TBLR1 complex *in vivo*.[15] *Xenopus* oocyte RNA injection system was used for this assay. mRNA for Flag-tagged PML-RARα (F-PML-RAR) was injected into oocytes, and immunoprecipitation (IP) using Flag antibody was performed. PML-RARα interacts with endogenous N-CoR/SMRT-TBLR1 complex in the absence of ATRA. In the presence of ATRA, this interaction was dissociated. Furthermore, chromatin immunoprecipitation assay (ChIP) was performed to show N-CoR/SMRT-TBLR1 interaction with target gene promoter DNA through PML-RAR. ChIP assay in oocyte system showed that endogenous N-CoR/SMRT-TBLR1 protein complex interact with the target promoter without ATRA, and the interaction was dissociated with ATRA. We then tried to use a dominant-negative protein, which interfere the TBLR1 interaction with N-CoR, to determine the TBLR1 function in the PML-RARα induced transcription repression. The transcription repression by PML-RARα was de-repressed by adding the dominant-negative protein. This experiment clearly suggests that TBLR1 is critical for the transcription repression by PML-RARα *in vivo*.

*Class I HDACs are the transcription repressor for PML-RARα*

Previously, it was reported that N-CoR-Sin3 complex interacts with unliganded PML-RARα. Furthermore, HDAC1 is also included in the N-CoR-Sin3 complex, and the histone de-acetylation activity of HDAC1 is required for the transcription repression by nuclear hormone receptors without ligands. It is reported that N-CoR-TBL1/R1 complex also contains one of class I HDACs, HDAC3.[6,12,17,19] We are currently focusing on this component, which may be utilized for the transcription repression by unliganded PML-RARα. First, we tried IP assay in human cell lines to show the interaction of PML-RARα with endogenous N-CoR and HDAC3. Flag-tagged PML-RARα was overexpressed in 293T cells, and Flag IP was performed. Endogenous N-CoR and HDAC3 interact with unliganded PML-RARα, and the interaction is dissociated by adding ATRA in dose dependent manner. Next, we tried ChIP assay to show the interaction of N-CoR-HDAC3 complex with endogenous target gene promoters through PML-RARα In the presence of PML-RARα, N-CoR-HDAC3 interact with target promoters without ligand, and the acetylation level of the neighboring histone tails were decreased. Using the RNA interference technique, HDAC3 knockdown was performed. In luciferase transcription assay using PML-RARα and its responding reporter plasmid that contains RAR responsive element sequences (RARE), siRNA for HDAC3 was introduced. If the expression of endogenous HDAC3 was down regulated by siRNA, PML-RARα dependent transcription repression

was significantly de-repressed. Furthermore, the expression levels of endogenous target genes that are repressed by PML-RARα over expression were de-repressed in the presence of siRNA for HDAC3. These results suggest that HDAC3 is also an important transcription co-repressor for the unliganded PML-RARα *in vivo*. It is also suggested that HDAC3 may contribute to the pathophysiologic role of APL cells.

*New molecular targets for the treatment of APL*

Together with these results, it is strongly suggested that N-CoR/SMRT-TBL1/R1-HDAC3 is one of an important transcription repressor protein complex for PML-RARα *in vivo*. To repress the function of these proteins induces transcription de-repression on the PML-RARα target gene expression. As is well known, ATRA acts as an elegant molecular targeting drug in APL cells to dissociate the N-CoR co-repressor complex from the aberrant chimeric transcription factor PML-RARα that interact with target gene promoters. Although recently, ATRA resistance caused by mutations in ligand binding domain of RAR and some chimeric transcription factors other than PML-RARα becomes a serious problem for the treatment of APL. We suppose that it may be useful for the treatment for such ATRA resistant APL patients, if we can interfere the function of the PML-RARα interacting transcription co-repressor proteins, such as N-CoR, TBL1/R1 and HDAC3. siRNA, inhibitor of the enzymes, anti-sense oligo, small peptide or protein which has dominant negative effect against those co-repressors are the good candidates for the therapy. We are now trying to use some of these to show the transcription de-repression and furthermore cell differentiation.

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