A functional SUMO-interacting motif in the transactivation domain of c-Myb regulates its myeloid transforming ability

Thomas Sæther [1*], Diwakar R. Pattabiraman [2*], Anne Hege Alm-Kristiansen [1], Linn Troye Vogt-Kielland [1], Thomas J. Gonda [2] and Odd Stokke Gabrielsen [1].

[1]*Department of Molecular Biosciences, University of Oslo, N-0316 Oslo, Norway.*
[2]*Diamantina Institute for Cancer, Immunology and Metabolic Medicine, University of Queensland, Brisbane, Queensland 4102, Australia.*

***** These authors contributed equally to this work

Correspondence: T. Sæther, Department of Molecular Biosciences, University of Oslo, P.O.Box 1041 Blindern, N-0316 Oslo, Norway.
E-mail: thomas.sather@imbv.uio.no
Tel.: +47 22 85 66 70

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ABSTRACT

c-Myb is an essential hematopoietic transcription factor that controls proliferation and differentiation of progenitors during blood cell development. Whereas sumoylation of the C-terminal regulatory domain (CRD) is known to have a major impact on the activity of c-Myb, no role for non-covalent binding of SUMO to c-Myb has been described. Based on the consensus SUMO-interacting motif (SIM) we identified and examined putative SIMs in human c-Myb. Interaction and reporter assays showed that the SIM in the in the transactivation domain of c-Myb (V_{267}NIV) is functional. This motif is necessary for c-Myb to be able to interact non-covalently with SUMO, preferentially SUMO2/3. Destroying the SUMO binding properties by mutation resulted in a large increase in the transactivation potential of c-Myb. Mutational analysis and overexpression of conjugation-defective SUMO argued against intramolecular repression caused by sumoylated CRD and in favour of SUMO-dependent repression in *trans*. Using both a myeloid cell line-based assay and a primary hematopoietic cell assay, we addressed the transforming abilities of SUMO binding and conjugation mutants. Interestingly, only loss of SUMO binding, and not SUMO conjugation, enhanced the myeloid transformational potential of c-Myb. c-Myb with the SIM mutated conferred a higher proliferative ability than the wild-type and caused an effective differentiation block. This establishes SUMO binding as a mechanism involved in modulating the transactivation activity of c-Myb, and responsible for keeping the transforming potential of the oncoprotein in check.

Keywords: c-Myb, SUMO, SUMO-interacting motif, myeloid, transformation, hematopoiesis

INTRODUCTION

SUMO (Small Ubiquitin-like modifier) has emerged as a major regulator of a host of cellular processes, and the number of targeted proteins is constantly growing (Geiss-Friedlander and Melchior 2007). Covalent conjugation of SUMO to nuclear factors mainly suppresses their activity and/or ability to synergize with other factors, alters their localization and interaction repertoire, or increases their stability (Gill 2005, Hay 2005, Kerscher et al 2006). With the increasing number of sumoylated targets, and the common assumption that the effects of SUMO must be mediated through protein interactions, the identification of a protein motif for non-covalent SUMO binding was awaited. In 2004 Song and co-workers showed using NMR that a small hydrophobic patch, V/I-X-V/I-V/I, was the minimal motif needed for SUMO interaction (Song et al 2004). This only partly matched a motif proposed earlier (Minty et al 2000). However, with the work of Hannich et al (2005) and Hecker et al (2006), the suggested consensus sequences were harmonized to V/I-X-V/I-V/I-a-a-a (a=acidic). Furthermore, the motif was shown to be able to bind to SUMO when reversed (Hecker et al 2006, Song et al 2005). The discovery of SUMO-interacting motifs (SIMs) has provided new insight into the interplay between sumoylation and SUMO binding, with the tumour suppressor PML as one of the best studied examples (Lallemand-Breitenbach et al 2008, Lin et al 2006, Shen et al 2006). The PML protein contains both sumoylation and SUMO-interacting motifs, and both motifs must be intact to form PML nuclear bodies (Shen et al 2006).

c-Myb is a sequence-specific transcription factor that controls proliferation and differentiation of early hematopoietic progenitor cells, as well as regulating similar processes in colonic crypts and neurogenic regions of the adult brain (Ramsay and Gonda 2008). The *MYB* locus is rearranged in several human neoplasias, with increased expression as a frequent outcome. This can be caused by translocation, leading to deregulation of the *MYB* gene, as in childhood T cell acute lymphoblastic leukemia (T-ALL) (Clappier et al 2007), or stabilisation of MYB mRNA, as in adenoid cystic carcinomas (ACC) of the breast, head and neck (Persson et al 2009). Local duplication of *MYB* has also been reported with another subgroup of T-ALL (Clappier et al 2007, Lahortiga et al 2007) and in a subgroup of acute myelomonocytic leukemia (AML) (Murati et al 2009). Thus, deregulation of c-Myb expression is associated with oncogenicity. Moreover, Myb's transcriptional regulatory activity is crucial for its transforming ability (Gonda et al 1989, Hu et al 1991, Lane et al 1990). Multiple co-factors like p300/CBP, Mi-2α, FLASH, and menin/MLL

engage in the regulation of the transactivational activity of c-Myb (Alm-Kristiansen et al 2008, Dai et al 1996, Jin et al 2010, Kasper et al 2002, Oelgeschlager et al 1996, Saether et al 2007). Recently, the importance of co-activation by p300 in myeloid transformation was highlighted employing a novel murine hematopoietic cell line transformation assay (Pattabiraman et al 2009). Moreover, the interaction between Myb and menin/MLL has been shown to be a critical driver in MLL-associated leukemogenesis (Jin et al 2010).

c-Myb becomes sumoylated in its C-terminal regulatory domain (CRD) at two sites, by both SUMO1 and SUMO2/3. This leads to a severe drop in the activity of c-Myb (Bies et al 2002, Dahle et al 2003, Sramko et al 2006). We have recently shown that this drop in activity is mainly due to silencing of a SUMO-regulated activation function in CRD, severely reducing the synergistic potential of c-Myb (Molvaersmyr et al 2010). In the oncogenic v-Myb protein, both SUMO conjugation sites are deleted and synergy control is lost. Whether loss of SUMO regulation is a central step in oncogenic activation of c-Myb has so far not been addressed.

In this paper we show that the transactivation potential of c-Myb is modulated not only through SUMO conjugation, but also through non-covalent SUMO binding. We have identified a functional SIM in the transactivation domain of c-Myb, which preferentially binds SUMO2/3. Abrogation of SUMO binding through mutation leads to an increase in c-Myb transactivational activity, mainly caused by lost repression in *trans*. Through the use of hematopoietic transformation assays, we show that loss of SUMO regulation can oncogenically activate c-Myb. However, only loss of SUMO binding, and not SUMO conjugation, unleashes the transforming potential of c-Myb.

RESULTS

c-Myb contains two putative SUMO interacting motifs

AMV v-Myb is one of two oncogenically activated forms of c-Myb known to cause acute leukemia in chickens. Deletions in the v-*myb* gene eliminate the C-terminal regulatory domain (CRD; Figure 1a), making v-Myb behave like an activated form of c-Myb in many assays with lost SUMO conjugation playing a key role (Dahle et al 2003). We recently realized that loss of SUMO conjugation sites might not be the only oncogenic alteration in v-Myb that relates to SUMO biology. Based on reports of a consensus SUMO-interacting motif (SIM; aaa-V/I-V/I-X-V/I/L and V/I-X-V/I-V/I-aaa) (Hecker et al 2006, Song et al 2004), we analysed the c-Myb sequence and found that it contains two putative SIMs; one in the R2 repeat in the DNA-binding domain (M1), and one in the N-terminal end of the transactivation domain (M2; Figures 1a and b). Both sites are evolutionarily well conserved, and remarkably, both are mutated in AMV v-Myb (Figure 1b). In fact, three of the ten oncogenic mutations in v-Myb are localized to the putative SIMs. The specificity of non-covalent SUMO1 versus SUMO2 binding lies in a stretch of negatively charged residues located directly N- or C-terminally of the core SIM motif (Hecker et al 2006). The fact that only M1 has such neighbouring residues implies that if functional, the c-Myb M1 would be a SUMO1-interacting motif, while M2 would mainly interact with SUMO2/3.

Destroying the putative SIM in the transactivation domain by mutations derepresses c-Myb

To investigate whether mutations in these potential SIMs would influence c-Myb activity, we made a set of mutants aiming to abrogate SUMO binding (Figure 1c). The mutants L106H and I_{267}NII were made to mimic the mutations found in v-Myb M1 and M2, respectively. However, since only the L106H mutation represented a deviation from the SIM consensus, additional mutations A_{103}AEA (wild type: V_{103}IEL) and A_{267}NAA (wild type: V_{267}NIV) were introduced to ensure a complete elimination of SUMO binding to these motifs.

We then performed effector-reporter assays using a c-Myb-responsive luciferase reporter. As can be seen in Figure 2a, the M1 mutant L106H had no effect, while the A_{103}AEA appeared to have a slight negative influence on c-Myb-mediated transactivation. On the other hand, the A_{267}NAA M2 mutant had a dramatic effect and activated c-Myb more than 13-fold relative to the wild-type. The activity of this mutant closely resembled that of the SUMO conjugation negative 2KR mutant (Dahle et al 2003), used as a positive control (Figure 2a). The v-Myb mimicking

mutation I_{267}NII had no such derepression effect; rather, it slightly lowered the activity of c-Myb. As can be seen in Figure 2b, none of these effects can be attributed to mutation-induced changes in protein expression levels. It therefore appears that the V_{267}NIV motif in c-Myb TAD has a strong suppressive function on activity, possibly mediated through the binding of SUMO, and that the A_{267}NAA mutant abrogates this suppression.

Human c-Myb binds SUMO via a SIM in the transactivation domain

To examine whether c-Myb was able to bind SUMO, we asked whether c-Myb could be pulled down from cell lysates using GST-SUMO1 and -SUMO2. This is currently one of the most stringent ways of detecting SUMO interactions, allowing both endogenous SUMO and SUMO-binding factors to compete for epitopes. SUMO, in general, binds to SIMs with affinities in the μM range (Hecker et al 2006), making the interaction somewhat difficult to detect. To avoid potential interference from SUMO moieties conjugated to c-Myb, we used a shortened version of human c-Myb (aa 1-409) where the CRD (harbouring the SUMO conjugation motifs) had been deleted. As can be seen in Figure 3a, c-Myb bound SUMO under these conditions. Furthermore, it seemed to interact more efficiently with SUMO2 than with SUMO1.

In order to determine which of the motifs might be responsible for the non-covalent binding of SUMO, we evaluated the different mutants in pull-down assays. Comparison of c-Myb wild-type with the M1 mutants (L106H and A_{103}AEA), did not reveal any difference in the affinity for SUMO (Figure 3b); both proteins interacted with SUMO2, and thus resembled the wild-type. In contrast, an obvious difference was observed when comparing the M2 mutants (A_{267}NAA and I_{267}NII) with the wild-type (Figure 3c). While the I_{267}NII mutant seemed to have retained the ability of c-Myb to bind SUMO, the A_{267}NAA mutant had lost this property. Importantly, we could also show that full-length c-Myb, like the shorter version, was retained with GST-SUMO2 (Figure 3d). Moreover, the M2 mutation ANAA lowered the affinity for SUMO2 substantially. Finally, we tried to study the interaction between SUMO and c-Myb, expressed at endogenous levels in erythroleukemia K562 cells. As expected, due to the low SIM K_d, only minute, but detectable, amounts of c-Myb was retained with GST-SUMO (Supplementary Figure 1).

The mutations found in the putative SIMs in v-Myb (L106H and INII) did not cause any major change in the SUMO binding properties of c-Myb (Figures 3c and d). In line with this v-Myb showed the same SUMO binding characteristics as c-Myb, including the preferred binding

to SUMO2 (Supplementary Figure 2). The same pattern emerged when using *in vitro* translated, [^{35}S]-labelled c-Myb as prey in pull-down assays (Supplementary Figure 3), but no SUMO preference was apparent in this setting. Some of these experiments were conducted in the presence or absence of ethidium bromide or DNA containing Myb-binding sites. None of these experiments indicated that an engaged DNA-binding domain changed the interaction between c-Myb and SUMO (data not shown).

Abrogating SUMO binding affects sumoylation of c-Myb

The evidence presented above show a physical, non-covalent interaction between SUMO and the c-Myb SIM, V_{267}NIV. Moreover, a derepression of c-Myb activity was observed with the A_{267}NAA mutant. Because the latter effect resembled that of the SUMO conjugation-disrupting 2KR mutations, we asked whether derepression by the ANAA mutation might be caused by SUMO conjugation being dependent on a functional SIM as previously shown for TDG, Daxx and SP100 (Knipscheer et al 2008, Lin et al 2006, Takahashi et al 2005). The two SIM mutants (A_{267}NAA and I_{267}NII) were expressed in CV-1 cells in absence or presence of the SUMO E2 conjugating enzyme Ubc9 or the SUMO E3 ligase PIASy increasing the relative amount of sumoylated c-Myb, and the sumoylation patterns were compared with those of c-Myb wild-type (two mono- + one disumoylated form) and 2KR (no sumoylated forms) (Figure 4). As can be seen in Figure 4a, the level of sumoylated c-Myb was lowered when the SIM consensus was lost (ANAA). The INII mutant on the other hand seemed to be sumoylated with the same efficiency as wild-type c-Myb. The reduced sumoylation efficiency seen with the ANAA mutant was persistent and could be seen when both E2 and E3 were overexpressed (Figure 4a). Nevertheless, c-Myb ANAA was modified, although at lower levels; this contrasts with c-Myb 2KR where SUMO conjugation was lost. We conclude that SUMO binding has a modulating effect on SUMO conjugation, without it being able to explain the high transactivational activity of the ANAA mutant.

Functional effects of altered SUMO interaction in the absence of SUMO conjugation

Previous studies have reported the existence of intramolecular interactions between the EVES domain and the N-terminal region of c-Myb (Dash et al 1996). Moreover, others have speculated that there might be an indirect contact between the CRD and the TAD (Dubendorff et al 1992,

Vorbrueggen et al 1994). Thus, we asked whether SUMO conjugates in the CRD and the SUMO-interacting motif in the TAD might interact to bridge these parts of c-Myb. We reasoned that if SUMO conjugation and SUMO binding were part of the same mechanism in c-Myb, destroying either one or both of these functions would lead to a similar enhancement of transactivation. As shown in Figures 2a and 5a, c-Myb A_{267}NAA and c-Myb 2KR possess almost identical activities. However, when both these mutations were introduced in the same construct (c-Myb ANAA 2KR), an additive increase in activity was observed (Figure 5a), arguing against these two mutants targeting the same mechanism. Even when the entire CRD was deleted, the A_{267}NAA mutation still increased the activity of c-Myb (Figure 5b). Thus, we conclude that the transactivation potential unleashed by the SIM mutation in c-Myb is disconnected from SUMO conjugation in the EVES domain. Even though the SUMO-interacting motif might be involved in recruiting or orienting components of the sumoylation apparatus, the SIM clearly also has a conjugation-independent function.

To make sure that what we had observed so far was also relevant for regulation of c-Myb-dependent activation of an endogenous chromatin-embedded gene, we tested the mutants for their ability to activate *mim-1* (Burk et al 1993, Ness et al 1993), using real-time PCR. Mutation of the SUMO-interacting motif in c-Myb resulted in significant increase in expression of *mim-1*, both alone and in combination with the 2KR mutation (Figure 5c). To ensure that the increased transactivational activity of the SUMO binding mutant was not caused by altered DNA-binding activity, we analyzed the promoter occupancy of c-Myb on the established target gene, *MYC*, using chromatin immunoprecipitation (ChIP). Neither the ANAA, nor the 2KR mutations, changed the ability of c-Myb to occupy the *MYC* promoter (Supplementary Figure 4). Together, this confirms a role for c-Myb SUMO binding in endogenous gene activation.

c-Myb binds SUMO in trans *in a SIM-dependent fashion*

Since our data obtained with double mutations and CRD-deletions did not support the hypothesis of intramolecular binding of SUMO, we predicted that the SIM had to bind SUMO in *trans* and that its repressive effect could be attributed to the recruitment of sumoylated, negatively acting co-factor(s). If so, it should be possible to titrate out this co-repressor with an excess of free SUMO, and thereby increase Myb activity. Thus, we expressed increasing amounts of non-conjugatable SUMO1 and -2 (mono-Gly in C-terminal) in the presence of our c-Myb mutants.

We expected the non-conjugatable SUMO to interfere mainly with SUMO binding, but also indirectly with conjugation, since the conjugation process is dependent on SUMO interactions (Reverter and Lima 2005, Tatham et al 2005). As can be seen in Figure 6a, co-expression of SUMO1-1G in the presence of wild-type c-Myb led to an increase in transactivation, while no significant change in activity was seen for c-Myb ANAA 2KR. The derepression of wild-type c-Myb was even more pronounced when titrating in increasing amounts of SUMO2-1G (Figure 6b), consistent with the observed preference for SUMO2. Interestingly, a reduced responsiveness was observed for c-Myb ANAA as well as for c-Myb 2KR, indicating that both proteins are partly uncoupled from SUMO-mediated repression. For c-Myb ANAA the reduced responsiveness is most probably due to lost SUMO binding, while the residual induction may be caused by the SUMO-1Gs interfering with sumoylation. For the 2KR mutant the situation is reversed, and the remaining ability to be induced must be due to SUMO-1Gs titrating out SUMO-binding factors.

As an alternative approach we also co-transfected our SUMO contact mutants together with plasmids expressing the SUMO-specific protease SENP1. The rationale was that SENP1 would desumoylate both c-Myb and other (co-)factors. SENP1 would therefore relax both SUMO binding- and conjugation-dependent repression of c-Myb activity. As shown in Figure 6c co-transfection led to an increase in activity for c-Myb wild-type, which was not seen with the catalytically dead SENP1 mutant. Moreover, both ANAA and 2KR mutants showed reduced potential for SENP1-induced activity. Importantly, only the double mutant, ANAA 2KR, was inert to the effects of desumoylation. Taken together these data strongly suggest that c-Myb binds SUMO in *trans*, probably in the form of a sumoylated co-repressor. This factor may be titrated out with free SUMO, with SUMO2 being the most efficient competitor due to its higher affinity for c-Myb.

We also investigated whether broken SUMO contacts might change the distribution of c-Myb. However, both in the absence and in the presence of PML IVa, a potential sumoylated co-repressor, inducing PML NBs, neither loss of SUMO-binding, nor SUMO-conjugation properties affected the subcellular localization of c-Myb or its recruitment to PML-NBs (Supplementary Figure 5).

Proliferation and differentiation properties of the SUMO contact mutants

To better understand the biological consequences of lost SUMO binding and conjugation, we investigated the transforming abilities of our c-Myb mutants. To this end we took advantage of a newly established murine hematopoietic cell line transformation assay (Pattabiraman et al 2009). FDB-1 is a factor-dependent cell line that proliferates in the presence of interleukin-3 (IL-3) and differentiates into granulocytes and macrophages within 7 days in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) (McCormack and Gonda 2000). c-Myb can, however, block GM-CSF-induced terminal differentiation of this cell line (Brown et al 2006), leading to continuous proliferation. We transduced FDB-1 cells with the GFP-tagged SUMO contact Myb mutants. The cells were then sorted for GFP expression before they were used to assay proliferation and differentiation properties. For control purposes the expression level and transactivational activity of the different Myb variants were validated by immunoblotting (Supplementary Figure 6) and effector-reporter assay (Supplementary Figure 7), respectively.

As described before, cells transduced with wild-type c-Myb and c-Myb CT3 proliferated continuously in the presence of GM-CSF (Figure 7a). As expected, the empty vector-transduced cells lost their capacity to proliferate following stimulation with GM-CSF. Interestingly, the cells transduced with c-Myb ANAA and ANAA 2KR showed very rapid proliferation (with the latter reproducibly slightly higher than the former) compared to either wild-type or CT3 Myb, while the cells transduced with c-Myb 2KR behaved much like the wild-type Myb-transduced cells (Figure 7a).

To examine the effects of the SUMO contact mutants on differentiation of FDB-1 cells, we assessed morphology by May-Grunwald-Giemsa staining following culture in GM-CSF for 7 days (Figures 7b and c). As reported earlier, c-Myb-transduced FDB-1 cells displayed a larger number of undifferentiated blast-like cells when compared empty vector-transduced cells (Figure 7b). Myeloblasts made up approximately 30% of the cells with both c-Myb wild-type and CT3, representing a ten-fold increase compared to the empty vector control. An even more pronounced differentiation block with up to 50% blasts was seen with the SUMO binding mutants ANAA and ANAA 2KR, while the SUMO conjugation-dead 2KR mutant once again generated a differentiation profile similar to c-Myb wild-type (Figure 7b). We also assayed the expression of the myeloid cell surface differentiation markers Gr-1 and Mac-1. As seen in Figures 7d and e, and Supplementary Figure 8, FDB-1 cells transduced with the ANAA and ANAA 2KR mutants

showed lower expression of Gr-1 and Mac-1 compared to Myb wild-type, CT3, and 2KR. These differences were, however, not statistically significant, but consistent with the morphological data (Figure 7b). To confirm the results from the cell line transformation assays, colony-forming assays were conducted using primary hematopoietic cells from murine bone marrow. As shown in Figure 8, c-Myb wild-type transduced cells formed around 40 colonies per 50000 cells plated, whereas c-Myb ANAA and ANAA 2KR, as well as CT3, formed almost four-fold more. In contrast c-Myb 2KR behaved like the wild-type. These results are consistent with the results from FDB-1 assays. Taken together, the transformation assay data imply that the SUMO binding mutants ANAA and ANAA-2KR, but not the SUMO conjugation mutant 2KR, possess enhanced transforming activity compared to wild-type c-Myb.

DISCUSSION

In this work we have identified a novel interaction of human c-Myb with SUMO, mediated by a SUMO-interacting motif (V_{267}NIV) close to the transactivation domain. This motif is involved in regulating the transactivational potential as well as modulating the SUMO modification of c-Myb. Most importantly, we show that SUMO binding is involved in dampening the transforming activity of this oncoprotein.

The functionality of this motif was assessed by two criteria: (1) c-Myb should show detectable binding affinity for SUMO, dependent on an intact SIM; and (2) removal of SIM by mutation should cause a change in the activity of c-Myb. The first criterion was addressed by GST pull-down assays with different SUMO isoforms and showed that c-Myb binds SUMO in a SIM-dependent fashion, with a clear preference for SUMO2/3 (Figure 3). Secondly, mutation of the SIM substantially increased Myb's transactivation potential (Figure 2). The determinant for SUMO isoform binding preference has been shown to lie in a stretch of negatively charged residues located directly N- or C-terminally of the SIM (Hecker et al 2006). Since the SIM in TAD only consisted of a hydrophobic core (closest acidic residue: +14), we reasoned that it would be a SUMO2/3-interacting motif, and indeed this was the case: In all the interaction assays we performed a preference for SUMO2 binding was observed (Figure 3). Still, SUMO1 binding might be functionally important when the modifier is conjugated to the appropriate factor, due to additional contact surfaces (Geiss-Friedlander and Melchior 2007).

Our data may explain previous observations on the effect of linker insertion mutagenesis in AMV v-*myb*. In studies performed to examine the correlation between transactivation and transformation by v-Myb, Lane and co-workers in fact generated one insertion mutant interfering with the SIM element as defined in this work [v-Myb 752; I_{202}NII→I_{202}NGPII (Lane et al 1990)]. Interestingly, this mutant was able to activate transcription 25-fold more efficiently than AMV v-Myb in QT6 cells (Chen and Lipsick 1993), suggesting that this might be due to loss of interaction with a unknown cellular inhibitor. In light of the present work their data may be explained by disruption of the SIM, hence SUMO binding.

Mechanistically, we would assume that the SIM acts through the interaction with a sumoylated protein, exerting a repressive effect on c-Myb. The most obvious alternative would be an intermolecular mechanism, where a SUMO-modified co-repressor would bind to the SIM. An alternative hypothesis would be that the SIM interacts intramolecularly with sumoylated c-

Myb CRD, leading to a repressed conformation of c-Myb. The second explanation would potentially substantiate the hypothesis of a fold-back mechanism involving the EVES domain and the N-terminal region in c-Myb (Dash et al 1996, Karafiat et al 2001) or the transactivation domain (Dubendorff et al 1992, Vorbrueggen et al 1994). Such intramolecular interactions have been hypothesized to conceal co-activator binding epitopes, thus lowering c-Myb activity. Indeed, the comparable activities of c-Myb 2KR and ANAA (Figures 2a and 5a) are consistent with a common mechanism, representing two ways of destroying the same intramolecular bridge. However, when introducing both mutations in the same construct, the transcriptional activity doubled (Figure 5a). Moreover, the ANAA mutant still activated when the CRD, including the SUMO-modified area of c-Myb, was deleted (Figure 5b). These data are not compatible with a SUMO-governed inhibitory fold-back mechanism in c-Myb, although they do not exclude the possibility of a fold-back mechanism not involving SUMO or not leading to activity changes. In light of these data it is interesting to notice that another type of crosstalk does occur. The SUMO-interacting motif in the TAD is involved in fine tuning the sumoylation of c-Myb in the EVES domain, such that, mutating the SIM reduces the sumoylation of c-Myb slightly (Figure 4a). Such interdependency has been shown for other SUMO targets, and reflects the fact that non-covalent binding of SUMO is an important mechanistic step in the conjugation reaction, orientating the SUMO moiety for optimal transfer (Reverter and Lima 2005, Tatham et al 2005).

Having ruled out the loss of intramolecular interaction as explanation of the increased transactivational activity of the SIM mutant, we addressed the possibility of intermolecular mechanisms. We reasoned that if a sumoylated factor was binding to c-Myb SIM, it should be possible to block this interaction by overexpressing non-conjugatable SUMO. Doing so we were able to increase the c-Myb activity, most likely by titrating out SUMO-binding repressors (Figure 6: wt vs. ANAA) and also by decreasing conjugation of SUMO to the sites in CRD through interference with the sumoylation apparatus (Figure 6: wt vs. 2KR). Thus, c-Myb is repressed in *trans* via the SIM, as well as via the SUMO moieties in EVES. The identification of SUMO contact-dependent co-repressors will be addressed in future work.

As the SUMO field has evolved, several diseases, including cancers, have been linked to perturbations in the SUMO system and/or disruption of sumoylation by mutations in substrate proteins (Hoeller et al 2006). Using hematopoietic transformation assays (Pattabiraman et al 2009), we examined the transforming abilities of the SUMO interaction mutants. Interestingly,

only the SUMO binding ANAA mutant seemed to be able to transform hematopoietic cells more effectively than wild-type c-Myb (Figure 7 and 8). In contrast, the non-conjugatable c-Myb 2KR had the same transforming potential as wild-type Myb, despite both ANAA and 2KR mutants being similarly highly active in transactivation assays. Furthermore, the double mutant ANAA 2KR was hyperactive in transactivation assays but was only marginally more active than ANAA in transforming the FDB1 cells (Figure 7). This is surprising because there is generally strong correlation between transactivation and transformation by c-Myb, e.g. (Hu et al 1991), and the importance of functional co-activation by CBP/p300 (Pattabiraman et al 2009) and menin/MLL (Jin et al 2010) has been shown. In cancers linked to aberrations involving the *MYB* locus, increased c-Myb dosage, and hence activity, seem to be a common theme (Clappier et al 2007, Lahortiga et al 2007, Persson et al 2009). Nevertheless, the 2KR and ANAA mutants described here seem to partially dissociate transactivation from transformation. Even though both types of SUMO contacts appear to restrict c-Myb activity to the same degree, they clearly differ when it comes to restricting Myb-dependent transformation. Thus, the putative repressor interacting with the SIM in TAD must play a particularly important role. Interestingly, the two factors shown to be necessary for driving transformation by c-Myb, p300 and MLL (Jin et al 2010, Pattabiraman et al 2009), both bind within the same region. Thus, our data on SUMO binding adds to the complexity and functional importance of the transactivation domain in c-Myb. It is becoming increasingly apparent that this domain is a sophisticated control region in which the critical functions of c-Myb are regulated through a multiplicity of interactions, balancing activation and transformation.

MATERIALS AND METHODS

Plasmids and antibodies

Information about the plasmids and antibodies used in this work is outlined in Supplementary Information

Protein expression and GST pull-down assay

GST and GST-SUMO fusion proteins were expressed in *E. coli* as previously described (Gabrielsen et al 1991). GST pull-down was performed with both COS-1 expressed and *in vitro* translated proteins as described in Supplementary information.

Cell culture and transfection, luciferase assays and immunoblotting

COS-1 and CV-1 cells were grown as described (Andersson et al 2003). For details on luciferase assays and immunoblotting see Supplementary information.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from transfected HD11 cells, reverse-transcribed and gene expression quantified as described in Supplementary Information

In vitro transformation assay

Assays were carried out as described in (Pattabiraman et al 2009) and as outlined in Supplementary Information

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Supplementary Information is available at Oncogene's website (http://www.nature.com/onc)

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FIGURE LEGENDS

Figure 1 c-Myb contains two putative SUMO-interacting motifs. (**a**) Schematic presentation of human c-Myb with its two potential SUMO-interacting motifs (SIMs), one residing in the R2 repeat of DBD and the other in the N-terminal part of TAD. DBD: DNA-binding domain, TAD: transactivation domain, CRD: C-terminal regulatory domain, R1, -2, -3: Myb repeat 1, 2 and 3, TP/CR: Thr- and Pro-rich conserved region, FAETL and EVES: motifs found within the assigned regions, LZ: putative leucine zipper. (**b**) Multiple sequence alignment of the areas harbouring the putative SIMs (boxed), using different mammalian c-Mybs. The acidic stretches close to the motifs are indicated by a solid line. AMV v-Myb is included for comparison. SUMO-interacting consensus motifs are included for clarity (Hecker et al 2006, Song et al 2005). (**c**) The SIM mutants used in this work. No change in amino acid residue is marked with a hyphen.

Figure 2 Destroying the putative SIM in the transactivation domain by mutations derepresses c-Myb. (**a**) CV-1 cells were transfected with a Myb-responsive *3×MRE(GG)-MYC* reporter plasmid (0.2 μg) and plasmids encoding full-length c-Myb wild-type, 2KR, AAEA, L106H, ANAA or INII in increasing amounts (0.2-0.4 μg). The results are presented as relative luciferase units (RLU). The results represent the mean RLU±SEM of at least three independent assays performed in triplicates. (**b**) CV-1 cells were transfected with plasmids encoding c-Myb-HA wild-type, 2KR, AAEA, L106H, ANAA or INII (0.2 μg). Cell lysates were subjected to SDS-PAGE and immunoblot analysis was performed using an anti-HA antibody.

Figure 3 Human c-Myb binds SUMO via a SIM in the transactivation domain. *In vitro* binding assays were performed in lysates from COS-1 cells transfected with (**a**) FLAG-tagged wild-type c-Myb (aa 1-409), (**b**) wild-type c-Myb (aa 1-409) and the M1 mutants AAEA and L106H, (**c**) wild-type c-Myb (aa 1-409) and the M2 mutants ANAA and INII, and (**d**) full-length c-Myb (aa 1-640), wild-type and ANAA. The lysate was incubated with comparable amounts of GST-SUMO-1 and -SUMO2 fusion proteins. The bound proteins were analyzed by SDS-PAGE and immunoblot analysis using an anti-FLAG antibody. 5 % of the input (total cell extract) used for the pull-down was loaded as reference. The amount of GST and GST fusion proteins was evaluated with Ponceau S red staining of the membrane after immunoblotting.

Figure 4 Abrogating SUMO binding affects sumoylation of c-Myb. CV-1 cells were transfected with plasmids encoding c-Myb-HA wild-type, ANAA, INII or 2KR (1.0 μg) alone or in combination with a small input of Ubc9 or PIASy expression plasmid (0.25 μg). Cells were scraped in cold PBS and lysed directly by sonication in SDS loading-buffer. The lysates were subjected to SDS-PAGE and immunoblot analysis using an anti-HA antibody. PIASy was visualised using an anti-T7 antibody.

Figure 5 Functional effects of altered SUMO interaction in the absence of SUMO conjugation. (**a**) CV-1 cells were transfected with a Myb-responsive *3×MRE(GG)-MYC* reporter plasmid (0.2 μg) and plasmids encoding full-length c-Myb wild-type, 2KR, ANAA or ANAA 2KR (0.4 μg). (**b**) CV-1 cells were transfected with a Myb-responsive *3×MRE(GG)-MYC* reporter plasmid (0.2 μg) and plasmids encoding c-Myb[1-409] wild-type, ANAA or INII in increasing amounts (0.2-0.4 μg). The results are presented as relative luciferase units (RLU). The results represent the mean RLU±SEM of at least three independent assays performed in triplicates. (**c**) Plasmids expressing c-Myb wild-type, 2KR, ANAA or ANAA 2KR were transfected into HD11 cells and total RNA was isolated. Activation of the endogenous Myb target gene, *mim-1*, was measured by quantitative real-time PCR using primers specific for the chicken *mim-1* and *HPRT* genes. The results are presented as *mim-1*/*HPRT* expression, and normalized to the ratio in empty vector transfected cells (set to 1.0). The results represent the mean ± SEM of two independent biological assays, each analyzed in duplicates. Expression of the different c-Myb mutants were evaluated by immunoblotting performed with an anti-HA antibody.

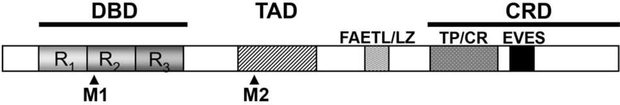
Figure 6 c-Myb is binding SUMO in *trans* in a SIM-dependent fashion. CV-1 cells were transfected with a Myb-responsive *3×MRE(GG)-MYC* reporter plasmid (0.2 μg) and plasmids encoding full-length c-Myb wild-type, ANAA, 2KR or ANAA 2KR (0.2 μg), in combination with increasing amounts (0-0.4 μg) of (**a**) SUMO1-1G or (**b**) SUMO2-1G (conjugation-deficient mutants). Increasing amounts of SUMO1-1G or SUMO2-1G (0–0.4 μg) were also transfected singularly together with the reporter. (**c**) c-Myb wild-type, ANAA, 2KR or ANAA 2KR (0.2 μg) were co-transfected with the SUMO protease SENP1 (0.2 μg) or SENP1 mutant (0.2 μg) as indicated. The results are presented as fold-induction of relative luciferase units (RLU), and the

activities of the different c-Myb proteins in the absence of SUMO is set to 1.0. The results represent the mean RLU±SEM of at least three independent assays performed in triplicates.

Figure 7 Proliferation and differentiation properties of the SUMO contact mutants. FDB-1 cells were transduced with empty vector, c-Myb wild-type, CT3, ANAA, 2KR or ANAA 2KR and grown in medium containing IL-3. (**a**) 20.000 cells transduced with each mutant were seeded at day 0 and supplied with GM-CFS. Cells were then counted over a period of 8 days. The results represent the mean cell number ± SEM. Experiments were carried out in triplicate and repeated thrice with consistent results. (**b**) Transduced FDB-1 cells were grown in IL-3 and/or GM-CSF-containing medium. After 7 days the cells were cytocentrifugated, the slides air-dried, fixed with methanol, and stained with May-Grunwald-Giemsa. (**c**) Approximately 500 cells per cytospin were scored based on their morphologic characteristics into myeloblasts, intermediate cells, and differentiated cells. Counts from at least three different cytospins were averaged. In addition 1×10^6 FDB-1cells were cells were stained simultaneously with (**d**) anti-Gr-1 APC and (**e**) anti-Mac-1 PE antibodies, washed, and fixed in 10% formalin. Stained cells were analyzed on a FACSCalibur flow cytometer. The results represent the mean value fluorescence ± SEM of three independent assays.

Figure 8 Primary cell colony transformation assays of c-Myb SUMO contacts mutants. Transforming ability of the mutants was measured by colony formation. Hemopoietic cells were isolated from bone marrow of adult C57Bl/6 mice and transduced with empty vector, c-Myb wild-type, CT3, ANAA, 2KR or ANAA 2KR. Colonies consisting of >50 cells were counted. The results are shown as scatter plots, and the bars represent the mean number of colonies ± SD of three separate experiments, carried out in triplicate.

a



b

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                   97     M1     109 265  M2                         292
c-Myb human      KEEDQR VIELV QK   LH VNIVNV PQPAAAAIQRHYNDEDPEKE
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c-Myb murine     KEEDQR VIELV QK   LH VNIVNV PQPAAAAIQRHYNDEDPEKE
c-Myb rattus     KEEDQR VIELV QK   LH VNIVNV PQPAAAAIQRHYNDEDPEKE
c-Myb chicken    KEEDQR VIELV QK   LH VNIVNV PQPAAAAIQRHYNDEDPEKE
c-Myb xenopus    KEEDQR VIELV HK   LR VNIVNV PQLATAPVQRHYNDEDPEKE
c-Myb oryzias    KEEDQR VIELV QK   LR FNILNI PQHGTAAIQRHYGEEDPEKE
                 ****** ***** :*   *: .**:*: ** .:*.:****.:******

v-Myb AMV        KEEDQR VIEHV QK   LH INIINV PQPAAAAIQRHYTDEDPEKE

Consensus: aaa-[V/I][V/I]X[V/I/L]  [V/I]X[V/I][V/I]-aaa
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c

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c-Myb A103AEA    KEEDQR AA-A- QK   LH ------ PQPAAAAIQRHYNDEDPEKE
c-Myb L106H      KEEDQR ---H- QK   LH ------ PQPAAAAIQRHYNDEDPEKE
c-Myb A267NAA    KEEDQR ----- QK   LH A-AA-- PQPAAAAIQRHYNDEDPEKE
c-Myb I267NII    KEEDQR ----- QK   LH I-II-- PQPAAAAIQRHYNDEDPEKE
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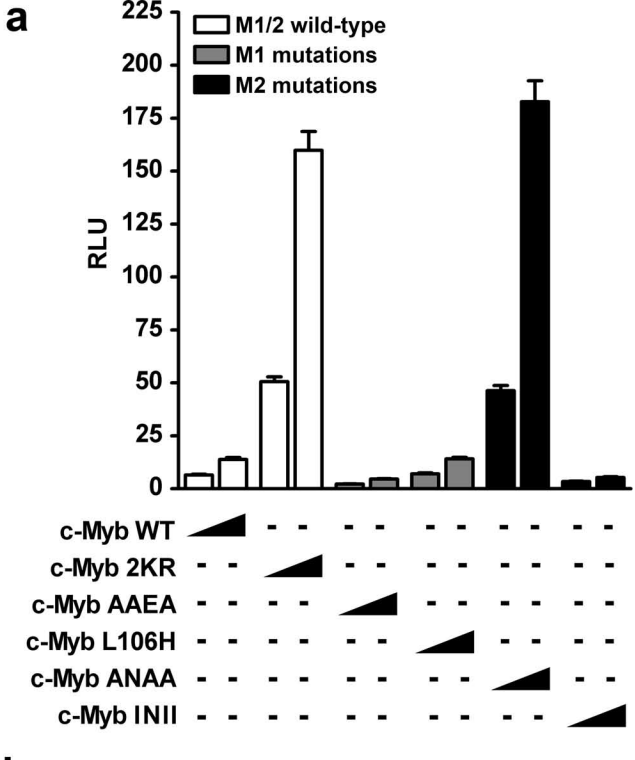
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M1 mutations
M2 mutations
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150
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75
50
25
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c-Myb 2KR
c-Myb AAEA
c-Myb L106H
c-Myb ANAA
c-Myb INII
b
IB: anti-HA
WT
2KR
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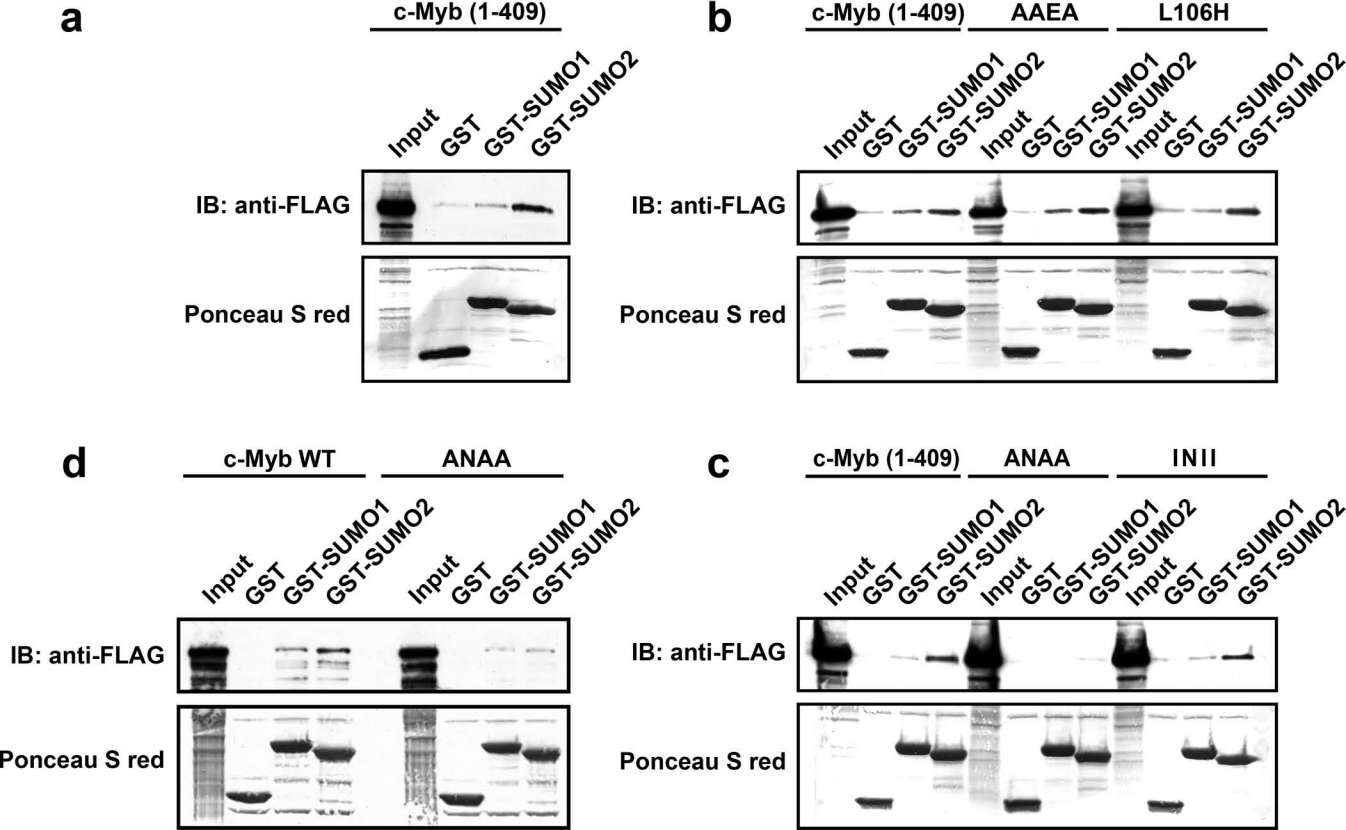
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Ponceau S red
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AAEA
L106H
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GST-SUMO2
IB: anti-FLAG
Ponceau S red
d
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ANAA
Input
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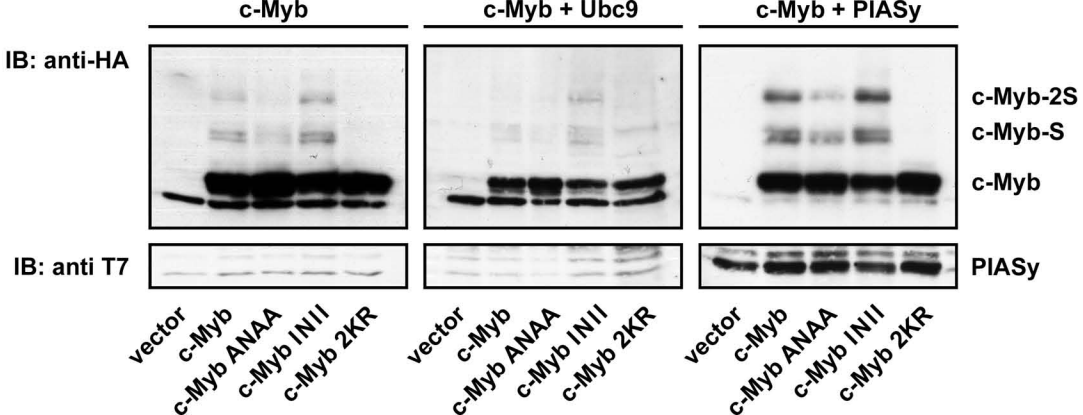
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PIASy
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vector
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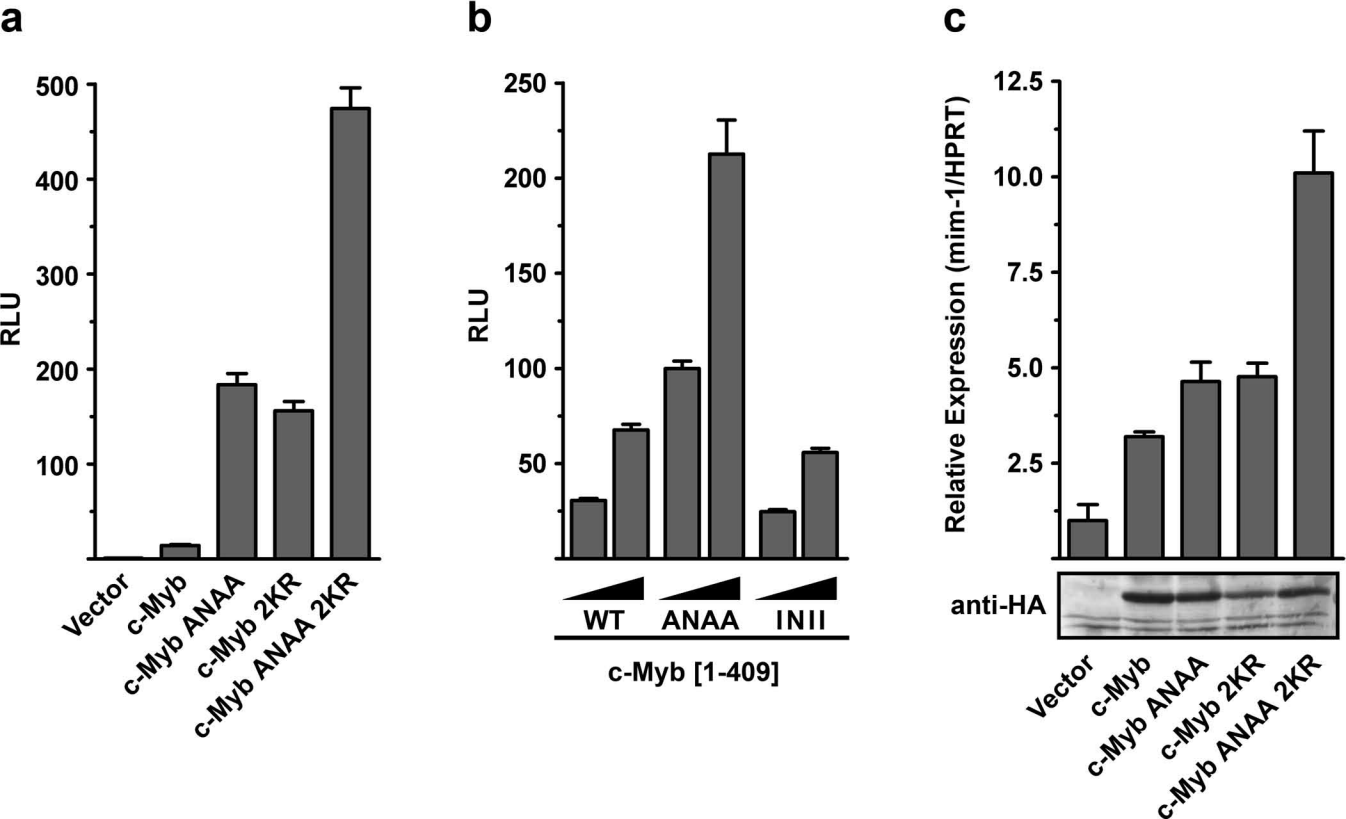
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200
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WT
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INII
c-Myb [1-409]
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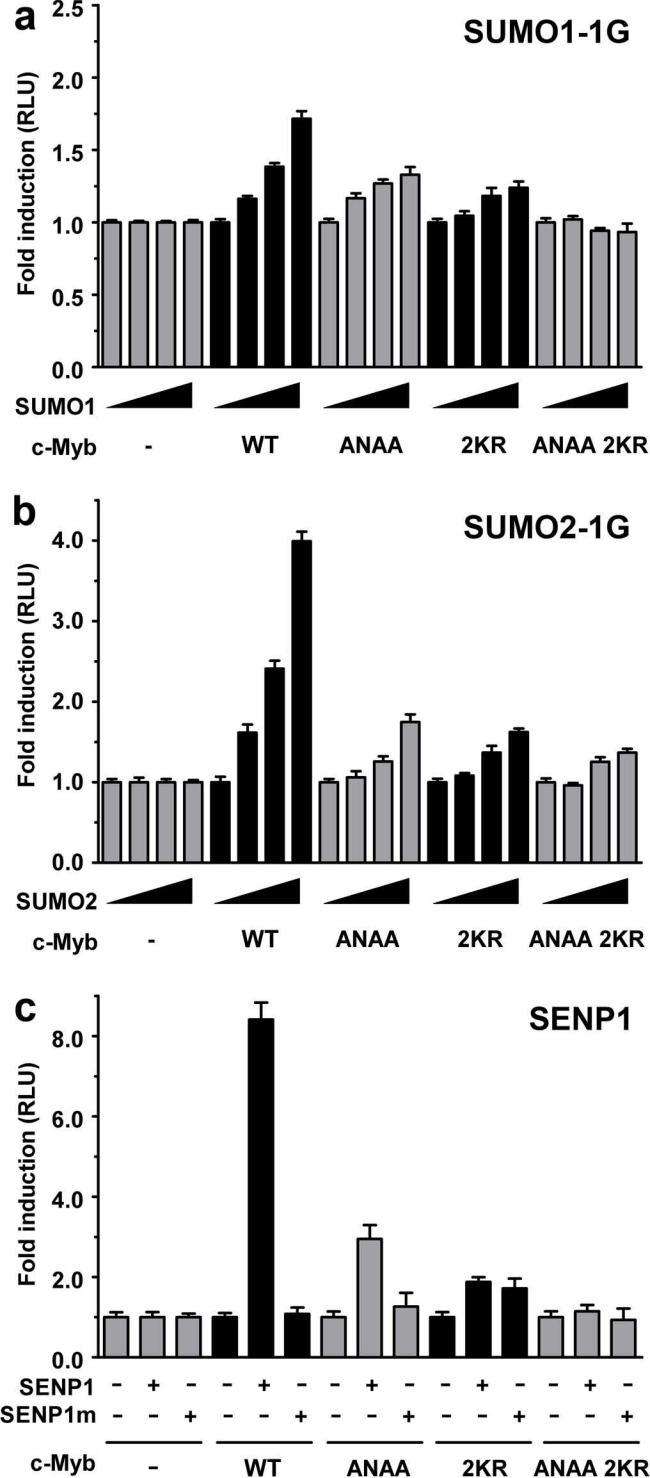
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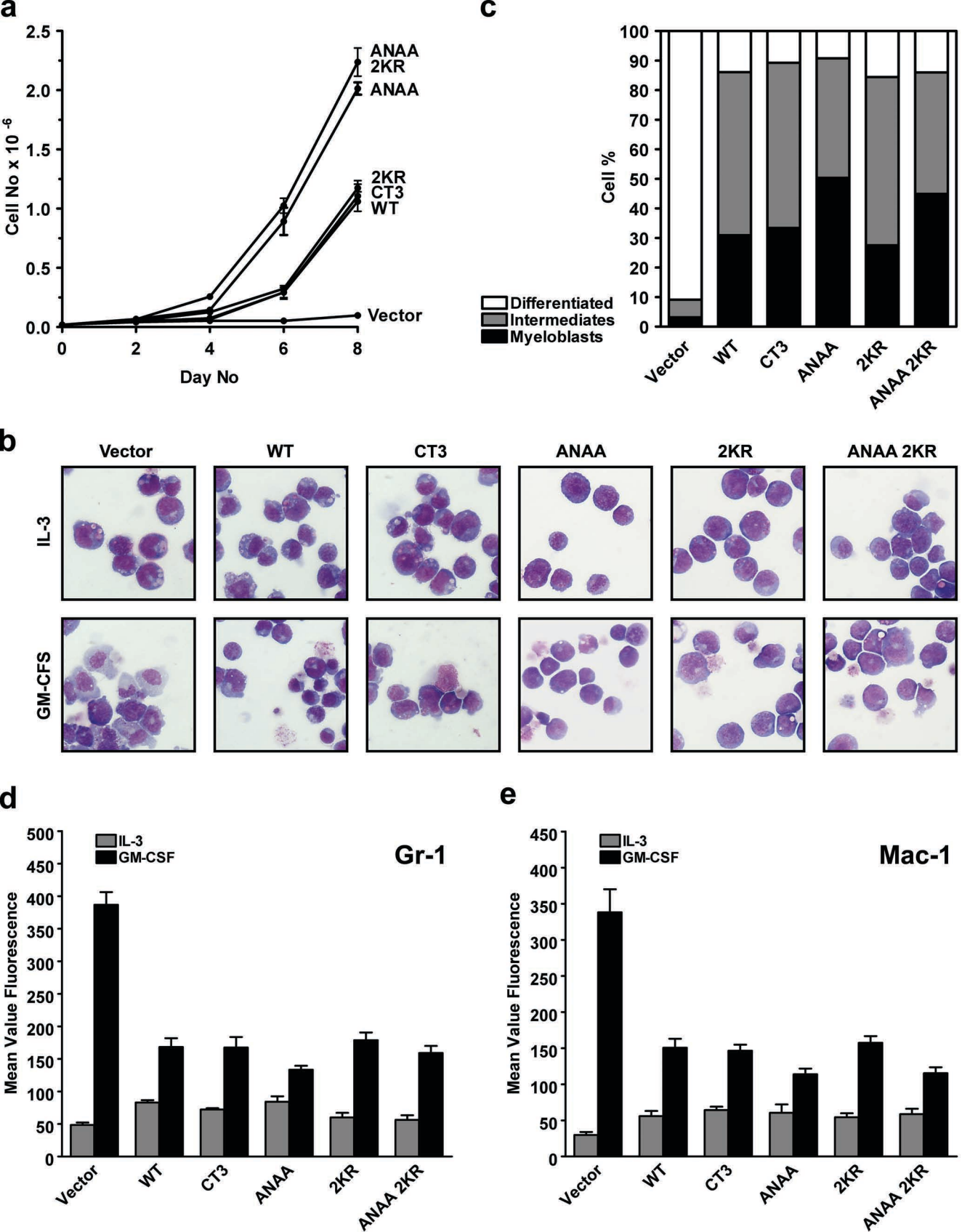
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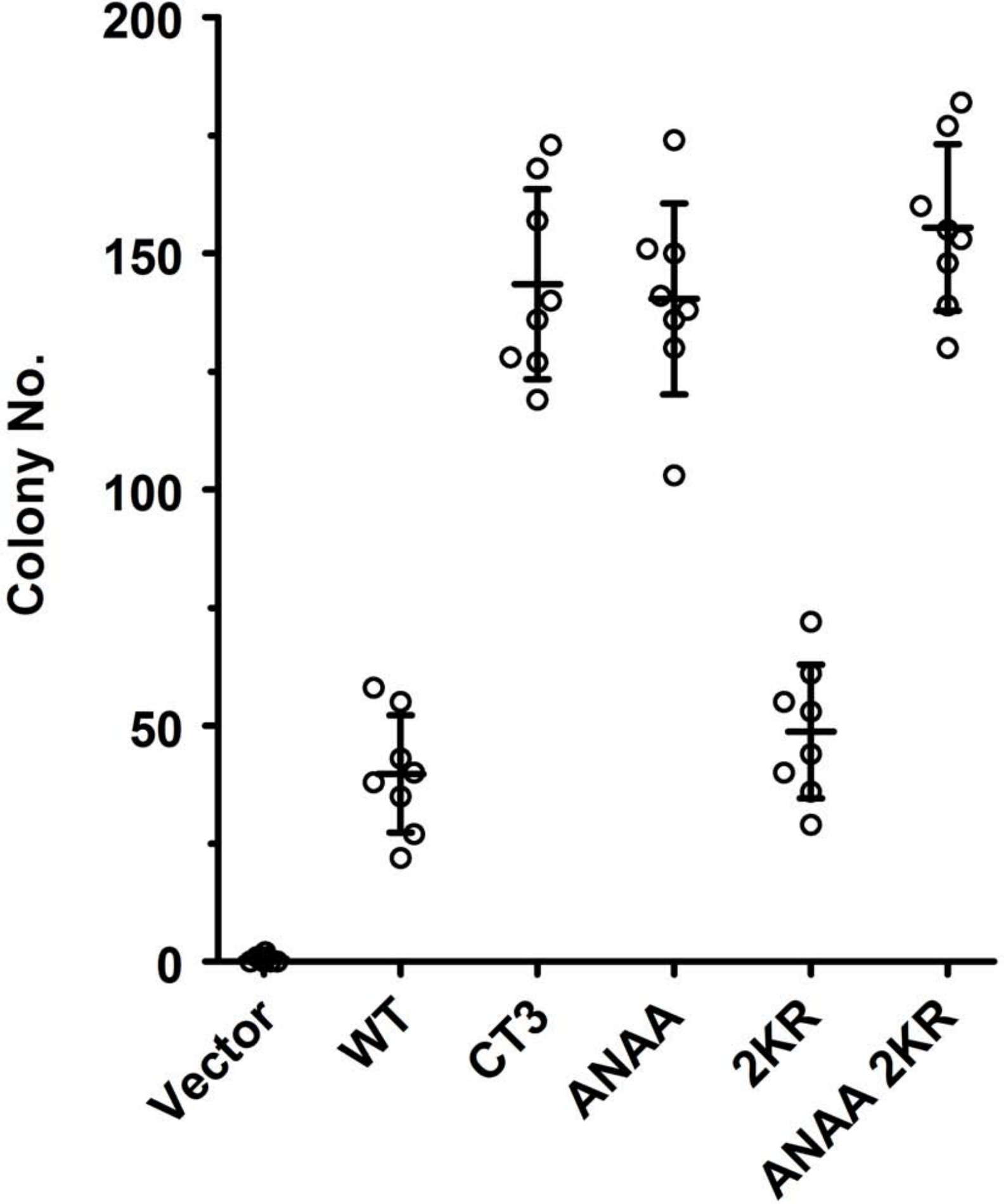
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