Ikaros is a key regulator of the hemo-lymphoid system in which it is presumed to function by both potentiating and repressing gene expression. Repression is mediated through two independent domains at the N and C terminus of the protein, both of which can independently recruit the corepressors Mi-2β, Sin3A, and Sin3B and the Class I histone deacetylases 1 and 2; the N-terminal domain can also associate with the corepressor CtBP. Here we describe a detailed dissection of these two domains and identify the minimal repression modules and the corepressor requirements for their activity. Based on these studies, we describe mutations in a full-length Ikaros protein that abrogate interactions with each of the identified corepressors and abolish the protein’s function as a repressor. Finally, we show that, barring CtBP, the Ikaros family members Aiolos, Helios, and Eos can associate with all of the identified corepressors of Ikaros including its newly identified interactors, Class II HDACs.

Ikaros (1) is the founding member of a Kruppel zinc finger family that includes Aiolos (2), Helios (3, 4) and Eos (5). The most full-length form of all these proteins is characterized by an N-terminal zinc finger DNA binding domain (6, 7) and two C-terminal zinc fingers related to the Drosophila gap protein hunchback, which are involved in interactions with self and with members of the family (8). Ikaros is comprised of seven exons from which at least eight isoforms (Ik1–8) can be generated through alternate splicing of exons 3–6 (6, 7). Exons 3–5 contain the DNA-binding zinc fingers, and their varying utilization results in Ikaros isoforms that can be distinguished by their ability to bind DNA (6, 7). The non-DNA-binding Ikaros isoforms, such as Ik6 and Ik7, interfere with the function of the DNA-binding forms and thereby can function as naturally occurring dominant-negative proteins (8). Mutations in the murine Ikaros and Aiolos loci, which prevent their expression and/or which exclusively generate dominant-negative proteins, have firmly established the importance of these genes in hemo-lymphoid development, differentiation, and homeostasis (9–12).

Ikaros and all its isoforms are capable of potent repression when tethered upstream of a promoter through a heterologous DNA-binding domain. Repression is effected through two domains found at the N and C terminus of the protein and, in some promoter contexts, depends upon the activity of histone deacetylases (HDACs) (13). It is widely presumed that HDACs contribute to repression by deacetylating lysines found at the N-terminal tails of histones H3 and H4 thereby facilitating a compact, closed chromatin configuration that is non-permissive for transcription (14).

HDAC proteins can be divided into at least three different classes based on sequence homology, expression, localization, and cofactor requirements: HDACs 1, 2, 3, and 8 comprise Class I, HDACs 4–7 belong to Class II, and SIRT 1–7 are members of Class III (15). Class I HDACs are nuclear and largely ubiquitous, whereas Class II HDACs are cytoplasmic (until they receive appropriate signals, whereupon they are imported to the nucleus) and more tissue-restricted (15). Class III proteins are NAD+-dependent histone deacetylases, which may also be involved in other histone modulating functions like ubiquitination (15).

In mammalian cells, Class I HDACs are primarily recruited to target genes through corepressor complexes containing either Sin3A, Sin3B (14, 16), Co-REST (17–19), or the chromatin remodeler Mi-2β (14, 16). Both the Sin3 and Mi-2β proteins interact with a core complex containing the Class I HDACs 1 and 2 and the Rb-associated proteins (RbAp), RbAp46 and RbAp48, but differ in the other proteins that they each associate with (14). Also, both the Sin3- and Mi-2β-containing complexes contain deacetylase and chromatin-remodeling activities (14, 20). The Sin3-HDAC complex is presumed to be recruited to euchromatic regions to transiently shut down genes, while the Mi-2β-containing nucleosome remodeling and deacetylation complex is thought to be involved in actively maintaining repression in heterochromatic regions. The Co-REST complex, which has only recently been described, does not contain any components in common with either the Sin3 or the Mi-2β complexes other than the Class I HDACs (17–19). In contrast to the Class I deacetylase complexes, Class II HDACs are found in association with the nuclear receptor corepressors, N-CoR or SMRT, and the Class I HDAC3 (21).

We have previously shown that both the N- and C-terminal repression domains of Ikaros can interact with the corepressors, Sin3A, Sin3B, Sin3BSF (a shorter isoform of Sin3B), Mi-2β, and Class I HDACs 1 & 2 (13, 22), while the N-terminal repression module can also interact with the corepressor CtBP through a PEDLS motif found in exon 2 (23). Here, we provide a detailed characterization of these two repression domains and identify the minimal regions within them that are required for repression and the corepressors that contribute to their function. We also describe mutations that prevent interactions with each of these corepressors and which can convert the strongest transcriptional repressor of the Ikaros isoforms, Ik6,
into a transcriptionally neutral protein. Finally, we show that, with the exception of CtBP, all Ikaros family members are capable of interactions with the identified corepressors of Ikaros, which suggests that they too may play a role in gene repression.

**EXPERIMENTAL PROCEDURES**

**Plasmids—Deletion mutations of the N- and C-terminal repression domains (IKD2 and IKD4) of Ikar1 and deletion mutants of Ikar6 were generated by cloning the relevant regions into the BXG1 vector, which encodes the Gal4 DNA binding domain (DBD) (amino acids 1–147) under the control of the SV40 promoter or into a CDM8Flag vector. CDM8-Ikar1, -Flag-Aio3, -Flag-Helios, -Flag-Eos (Daedalus), -Mi2β, -MT-Sin3A, -HA-Sin3B, pCMV2-FlagIk1, -FlagIk1 cm, BXG1-Ik6, and the reporter GSTkCAT have been previously reported (13, 23). Flag-HDAC3 was provided by Dr. E. Seto, MT-HDAC4 was provided by Dr. M. Lazar, and HA-HDAC5 and -HDAC7 were provided by Dr. R. Evans.**

**Transfections—**293T and NIH3T3 cell lines were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (HyClone). Transfections of these cell lines were carried out using the HBS-CAp04 method. For repression assays, 1 μg of the Gal4 fusion plasmid, 10 μg of the Gal4 reporter plasmid, and 0.5 μg of the pXGH5 green fluorescent protein (GFP) expression plasmid were used. Twenty-four hours after transfection, cells were fed with fresh media, and 18–24 h later cells were harvested and processed for CAT assays as described (18). Growth hormone assays were done as recommended by the manufacturer (Nichols Institute). Transfections were typically performed in duplicate and repeated between three and six times.

**Immunoprecipitation and Western Analysis—**Whole cell extracts from 293T cells transfected with the relevant plasmids were prepared as previously described (8) and precleared using protein-Gagarose beads (Roche Molecular Biochemicals). The precleared extracts were incubated with the antibody of interest or the relevant isotype control on ice for 1 h. 30 μl of protein-G beads were then added to the extract, and the extracts were rotated overnight. The beads were collected by centrifugation and washed four times with T5 buffer (150 mM NaCl, 20 mM Tris, pH 7.5) and treated with SDS sample buffer, boiled at 95 °C for 15 min, and loaded on an SDS-polyacrylamide gel along with 8–10% of the cell extract used for the immunoprecipitation. The proteins were transferred to a nitrocellulose membrane, probed with the relevant antibody, and examined by autoradiography with ECL (Amersham Biosciences). Antibodies used were: Myc tag (MT) (Roche Molecular Biochemicals), HA (BalbCO), FLAG M2 (Sigma), Gal4, Sin3B (Santa Cruz), HDAC2 (Zymed Laboratories Inc.), and anti-Ikaros and Mi-2, which have been previously described (22).

**RESULTS**

**Dissection of the Two Repression Domains of Ikaros—**We have previously shown the existence of two repression domains in Ikaros namely, IKD2 and IKD4 (see Fig. 1A, constructs 2 and 14 respectively) (13). To delineate the minimal regions capable of repression within these two domains, we constructed a series of N- and C-terminal deletions in the context of the Gal4 DBD. Each of these constructs was first tested for expression (Fig. 1B) and then transfected together with a thymidine kinase promoter-CAT reporter engineered with five Gal4 binding sites (G5kCAT) into NIH3T3 cells to assay the ability of the encoded proteins to repress transcription.

The N-terminal repression domain, IKD2, and all of its deletion derivatives were capable of repression, albeit to differing levels (Fig. 1A, constructs 2–11). Repression was maximal when all four N-terminal zinc fingers were present; however, deleting all four N-terminal zinc fingers still permitted the resulting region to repress transcription (Fig. 1A, construct 8). In contrast, not all derivatives of the C-terminal repression domain function as repressors (Fig. 1A, constructs 12–13); the region containing the two C-terminal zinc fingers was essential for repression. Mutation of these zinc fingers, either by point mutations that alter their ability to dimerize or by deletion of one or both fingers, reduces or abolishes the repressive function of this region (Fig. 1A, compare construct 12 to 13; 14 to 15 and 16; and 17 to 18 and 19). However, a region comprising the C-terminal zinc fingers was, by itself, only capable of modest repression (Fig. 1A, construct 20). This region may need to operate in the context of a larger domain and thus not function effectively when directly tethered to the Gal4 DBD.

In summary, the N- and C-terminal repression domains of Ikaros can be distinguished by the behavior of their deletion derivatives; the former is comprised of several smaller apparently redundant repression modules, whereas the latter is critically dependent on one region that contains the C-terminal zinc fingers.

**Corepressor Interactions Required for Repression by Ikaros Domains—**In previous reports we have shown that Ikaros is a component of the Mi-2/nucleosome remodeling and deacetylation complex and Sin3-HDAC chromatin-modifying complexes in lymphocytes (13, 22); in addition, it also interacts with the corepressor CtBP (23). We set out to examine whether these interactions were responsible for the activity of the Ikaros N- and C-terminal repression regions.

The N-terminal Ikaros repression domain, IKD2, can interact with the corepressors, Sin3A (Fig. 2A), Sin3BSF (B), Mi-2β (C), HDAC2 (D), and CtBP (23). C-terminal deletions of this domain altered its interactions with the Mi-2β and Sin3 proteins; in particular, removal of the third and fourth zinc fingers severely compromised interactions with Mi-2β (Figs. 1A and 2C, compare IKD2B to IKD2C) and Sin3 proteins (Figs. 1A and 2A, A and B, compare IKD2 to IKD2C and IKD2D). N-terminal deletions of IKD2 that left all four zinc fingers intact had no effect on Mi-2β (Figs. 1A and 2C, IKD13 and -14) but did have a small effect on Sin3 interactions (Fig. 2A, IKD14). Interestingly, the deletion mutants, which were unable to interact with Sin3 and Mi-2β, were still capable of repression (see Fig. 1A, IKD2C; Fig. 2D, N1, N2, and IKD15). This may be due to their ability to still interact with HDAC2 (Figs. 1A and 2D) and CtBP1 (Fig. 1A). CtBP1 was previously shown to interact with Ikaros through a PEDLS motif (23), which is located upstream of the Sin3 and Mi-2β interaction domains. Nonetheless, deletion of the CtBP interaction motif in the context of a region that contained the Sin3 and Mi-2β interaction interfaces had minimal effects on the repressive activity of the resulting region (Fig. 1A, IKD13, IKD14). Finally, a deletion designed to abrogate CtBP, Sin3, and Mi-2β interactions at the N-terminal region of Ikaros was still capable of repression (Fig. 1A, IKD15); however, this deletion mutant was still capable of interactions with HDAC2 (Fig. 1A, construct 11; Fig. 2D).

The C-terminal repression domain, IKD4, also interacted with Sin3 (Fig. 2, A and B) and Mi-2β and HDAC2 (Fig. 2D) but not with CtBP. A 97-amino acid module spanning residues 361–457, located upstream of the self-interaction zinc fingers (Fig. 2B, IKD8), could support Sin3 interactions; interestingly, this region was incapable of repression (Fig. 1A, constructs 13, 16, and 19). The smallest region capable of interaction with the chromatin remodeler, Mi-2β, at the C terminus was mapped previously by yeast two-hybrid assays and spans amino acids 457–518, which contain the two self-interaction fingers of Ikaros; deletion mutants of this region are unable to repress. This is in contrast to the N-terminal subregions that are unable to interact with Mi-2β but which are still capable of repression (Fig. 1A, compare construct 4 to 5 and 7). HDAC2 interactions at the C terminus resemble those of Mi-2β but not Sin3: HDAC2 interacts with IKD5, which interacts with Mi-2β but not with IKD8, which interacts with Sin3 (Fig. 1A). Thus, HDAC2 may be recruited to the C terminus of Ikaros through Mi-2β and not Sin3.

Taken together these data indicate that Ikaros contains several distinct interaction interfaces for corepressor molecules (shown over the Ikaros schematic in Fig. 1A). The N-terminal
Fig. 1. Dissection of the two repression domains of Ikaros. A, schematic representation of Ik1 and its subregions. Exons (E) are indicated as horizontal rectangles, and zinc fingers by vertical rectangles. The absence of vertical rectangles for construct 18 indicates that its zinc fingers have been mutated to prevent dimerization. The repression supported by each subregion is tabulated adjacent to its diagrammatic representation. The amino acids delineating each subregion are indicated. NIH3T3 cells were transfected with the indicated Gal4 constructs (BXG1, 1 μg), the reporter G5tkCAT (10 μg), and pXGH5 (1 μg), a growth hormone-expressing vector to account for differences in transfection efficiency. Fold repression was calculated by dividing the CAT activity supported by each BXG1-Ikaros subregion (measured as cpm) by the CAT activity of the BXG1 vector alone. The fold repression values are the average of transfections done in duplicate six different times. The variation between independent experiments was less than 20%. Also indicated in the table are the results of interaction mapping studies of each of these subregions with the corepressors of Ikaros: Sin3A, Sin3BSF, Mi-2, HDAC2, and CtBP, which were performed as described in Fig. 2. Binding is indicated as +, and its lack thereof as −. n.d. stands for not done. Please note that binding should not be taken to mean strength of interaction. The asterisk (*) identifies those interaction assays that were performed in yeast. The binding data for CtBP1 is based on previously published data (23). The minimal interaction interfaces for the tested corepressors, gleaned from these studies, is indicated above the schematic of Ik1, whereas the functional domains are indicated below. B, 293T cells were transfected with each of the Gal4-tagged Ikaros subregions diagrammed in A. Whole cell lysates were prepared and analyzed by immunoblot analysis using Gal4 antibodies to detect expression. The numbers listed above the blot correspond to the number assigned to each subregion in A. The arrowhead identifies a cross-reacting band.
half of the Ikaros protein contains sites that promote binding of Sin3, Mi-2β, HDAC2, and CtBP. Mutations that ablate any one of these corepressor interaction interfaces compromise, but do not ablate, the repression potential of this region. Thus there is an apparent redundancy for repressor function in this part of the molecule. In contrast, although the C-terminal half of the Ikaros protein can also bind Sin3, Mi-2β, and HDAC2, repression activity appears to rely predominantly on the C-terminal interaction zinc fingers, which are part of the Mi-2β and HDAC2 but not the Sin3 interaction interface.

Reversal of Ik6 Repression Relies on the Abrogation of Mi-2β, HDAC2, and CtBP Interactions—Having identified regions of...
the Ikaros protein with the potential to repress and interact with its known corepressors, we set out to determine the role of a subset of these putative interaction interfaces in the context of the smallest full-length Ikaros protein, the Ik6 isoform. Ik6 represents a subclass of Ikaros isoforms that lack a DNA binding domain and which have been implicated in leukemogenesis (10). Therefore, delineating the transcriptional functions of this Ikaros isoform could be important in dissecting its role in leukemogenesis. Interestingly, Ik6 is the strongest repressor of all Ikaros isoforms when recruited to DNA through a heterologous DNA binding domain (13).

A series of Ik6 deletion mutants were generated (Fig. 3C) and tested for their ability to interact with the corepressors of Ikaros. Deletion of 33 amino acids within the 97-amino acid region identified as the C-terminal Sin3 interaction module (Fig. 1A, IKD8), abolished interactions of Ik6 with Sin3A, Sin3B, and Sin3BSF without affecting interactions with Mi-2β or HDAC2 (Fig. 3, A and C, M6, and data not shown). A different deletion in the region encompassing the two C-terminal zinc fingers of Ikaros abolished interactions with Mi-2β and dramatically reduced interactions with HDAC2 (Fig. 3B and C, M17). We have previously shown that a mutation of the PEDLS motif at the N terminus of Ikaros ablates its interactions with the corepressor CtBP (23).

Given that Ik6 lacks a DNA binding domain, its corepressor interaction mutants were tested for their ability to repress transcription in the context of the Gal4 DBD. The CtBP interaction mutant (M1) was impaired by over 50% compared with wild type in its ability to repress transcription, while the Sin3 interaction mutant (M6) was only modestly affected (Fig. 4). A mutation that abolished Mi-2β binding and significantly altered HDAC2 association (M17) could repress to only 20% of wild type levels (Fig. 4). When this mutation was combined with one that prevented CtBP interaction (M18), repression was completely abolished (Fig. 4). Thus, Mi-2β, HDAC2, and CtBP appear to play key roles in Ik6-mediated repression.

Mutations that prevent Ikaros interactions with self and family, M12, M14, and M15, also decreased repression. As these mutations do not appear to affect interactions with Mi-2β, HDAC2, and presumably CtBP (Fig. 3, B and C), decreased repression may result from an altered Ik6/corepressor configuration that prevents efficient repression, or alternatively, these mutants may be defective in interactions with other yet-to-be identified Ikaros corepressors.

Corepressor Interactions with Ikaros Family Members— Ikaros is the prototype of a family of transcription factors that exhibit a restricted pattern of expression in hemopoietic, neuronal, and epithelial tissues (2–5). The non-hemopoietic Ikaros family members may play a similar role to Ikaros in the development systems where they are present. We therefore examined whether the Ikaros corepressor interactions were conserved between its family members. Aiolos, Helios, and Eos were expressed either alone or in combination with a subset of corepressors. Using immunoprecipitation assays, we found that all Ikaros family members could interact with ectopically expressed Sin3A (Fig. 5A) and Sin3B (B) and endogenous Mi-2β (C) and Class I HDAC2 (D).

Since our initial description of Ikaros interactions with Class I HDACs, other classes of HDACs have been identified that also function in repression. Ikaros and all its family members were tested for interactions with Class II HDACs. All Ikaros family members interacted best with HDAC5 (Fig. 5E) and to a lesser but differing extent with HDACs 4 and 7; Helios interactions with these HDACs are almost undetectable, whereas Eos interacts with them most strongly among the Ikaros family (Fig. 5E). We also compared binding of Ikaros with the two classes of HDACs. Ik1 was co-transfected with the Class I HDACs 1 and 3 or the Class II HDACs 4, 5, and 7. Immunoprecipitation assays revealed that Ikaros interacted more strongly with Class I HDACs compared with any of the Class II members (Fig. 5F).

In summary, Ikaros family members can interact with all of the identified corepressors of Ikaros except CtBP. Thus, they too may play a role in gene repression. In agreement with this expectation, both Aiolos (13) and Eos (27), like Ikaros, have been shown to be capable of repression.

**DISCUSSION**

We have previously shown that Ikaros can function as a potent repressor when recruited to promoters through a heterologous DNA binding domain (13). This function, at least in certain contexts, is mediated through the action of histone deacetylases (13). Consistent with this observation, Ikaros has been shown to be associated with at least two distinct HDAC complexes containing the corepressors Mi-2β and Sin3, respectively (13, 22). Ikaros also interacts with the corepressor CtBP, which despite associating with HDACs, can repress transcription in an HDAC activity-independent manner (23). In an effort to determine the role of these corepressor interactions in Ikaros-mediated repression, we undertook a detailed molecular analysis of the repression domains of Ikaros. These studies have allowed the delineation of the minimal repression modules of Ikaros and the corepressor interactions required for their function. Integrating these findings in the context of the potent transcriptional repressor, Ik6, we report the successful construction of the first repression-defective Ikaros protein.

A systematic dissection of the two Ikaros repression domains has allowed the identification of interaction interfaces on Ikaros for its corepressors (see Fig. 1A). We have previously shown that CtBP1 binds Ikaros through a PEDLS motif within the N-terminal repression domain (23). Unlike CtBP, the Sin3, Mi-2β, and HDAC2 corepressors associate with Ikaros through modules in both the N- and C-terminal repression domains. At the N terminus, Sin3 and Mi-2β interact with a region spanning the DNA-binding zinc fingers, while HDAC2 associates with all N-terminal subregions tested to date (albeit to differing levels) indicating that it can directly bind or be recruited to Ikaros through independent strategies. At the C terminus, Mi-2β and HDAC2 interactions with Ikaros are dependent on the region spanning the zinc fingers involved in dimerization, whereas Sin3 binds a region immediately adjacent to this region. Interestingly, unlike Ikaros and its family members, both Mi-2β and HDAC2 do not require the dimerization function of the C-terminal zinc fingers to associate with Ikaros. The interactions of Mi-2β, HDAC2, and Sin3 with the region spanning the DBD of Ikaros and of Mi-2β and HDAC2 with the dimerization module open up the possibility that they may be involved in modulating the functions of these domains.

In agreement with the corepressor interaction modules described above, the N-terminal repression domain, IKD2, can be broken into smaller subregions, all of which can repress transcription, whereas the C-terminal domain, IKD4, can only function effectively when the region spanning the two C-terminal zinc fingers is intact. Interestingly, the tricho-rhino-phalangeal syndrome protein 1 (TRPS1), which contains two zinc fingers with extensive homology to those at the C terminus of Ikaros (24), also cannot repress transcription when this domain is deleted (25). Thus, the C-terminal zinc finger domain, which is found in proteins as early as *Drosophila* hunchback (26) and in proteins otherwise unrelated to Ikaros such as Pegasus (27) and TRPS1 (24), appears to be an evolutionarily conserved module involved in dimerization as well as gene repression.

Repression mediated by each subregion of the two repression
FIG. 3. Generation of mutations in Ik6 that abrogate corepressor interactions. A, 293T cells were transfected with wild type Ik6 and Ik6 mutants (diagrammatically represented in C) alone or in combination with MT-Sin3A or HA-Sin3B (long form). Whole cell lysates prepared from the transfected cells were immunoprecipitated (IP) with antibodies raised to Ikaros or isotype control antibody (mouse IgG, mIgG) and immunoblotted (IB) with the indicated antibodies to test for binding the ectopically expressed Sin3 or the endogenous Mi-2β and HDAC2 proteins. Blots were stripped and reprobed with Ikaros antibodies to confirm that each of the proteins were made and precipitated. B, wild type Ik6 and deletion mutants (see C) were transfected into 293T cells. Whole cell lysates were immunoprecipitated as described in A. Immunoblots were probed sequentially with antibodies to detect interactions between Ikaros and endogenous Mi-2 and HDAC2. C, a diagrammatic representation of the Ik6 proteins used for mapping corepressor interaction mutants alongside the results from this study. The horizontal rectangles indicate exons (E), whereas the vertical rectangles indicate the two C-terminal zinc fingers. The lack of a vertical rectangle indicates point mutations in the corresponding finger that convert the two cysteines of the corresponding finger to glycines, which abrogate interactions with Ikaros or its family. The asterisk identifies a DL → AS mutation in exon2 of Ikaros that prevents its association with the corepressor CtBP. + signifies interaction, – signifies a significant reduction in interaction compared with the wild type, – indicates lack of interaction, and n.d. indicates that the interaction has not been tested. D, a schematic representation of the data in C.
domains of Ikaros correlated with their interactions with at least one of the corepressors of Ikaros. In the context of the N-terminal repression domain, subregions that could not interact with CtBP, Sin3, or Mi-2/H9252 individually or in combination were not defective for repression; this can be explained by the fact that HDAC2 could interact with all tested N-terminal subregions. Alternatively, or in addition, unidentified corepressors may contribute to the redundancy of repression function that exists for this domain. In contrast, subregions at the C terminus that could not associate with Mi-2/H9252 were defective for repression. Thus, the N- and C-terminal repression domains of Ikaros have significantly different corepressor requirements for repressive function.

Another interesting observation from these studies was that several subregions that interacted with the corepressor Sin3 were incapable of supporting repression (IKD11, IKD4B, IKD8). This suggests that corepressor interaction per se is insufficient, in some cases, for repression; in these instances the corepressors may need to be assembled into a higher order complex (for which other regions are presumably required) to effectively turn off gene expression. Alternatively, Sin3 may play a role different from transcriptional repression in the context of Ikaros.

Based on these collective findings, we targeted mutations in the smallest and most potent Ikaros isoform, Ik6, to construct a repression-defective Ikaros protein. A mutation in Ik6 that prevented the corepressor Sin3 from binding Ikaros had only minimal effects on repression. In contrast, a mutation that abrogated CtBP binding reduced repression by 50%. A deletion of the C-terminal zinc fingers, which abolished Mi-2 binding and also dramatically reduced HDAC2 association, brought repression down to 20% of wild type levels. When this mutation was coupled with one that prevented CtBP interactions, the resulting protein was completely incapable of repressing transcription. Taken together, these data highlight the importance of the dimerization module for Ik6 to repress transcription and also suggests that this isoform likely effects repression through the combinatorial action of Mi-2/H9252, HDAC2, and CtBP.

In contrast to the results obtained for Ik6, mutations of the C-terminal zinc fingers in the context of the most full-length Ikaros isoform, Ik1, had no significant impact on repression (13). This may be explained by the presence, in the full-length protein, of the additional N-terminal repression domain, which can interact with all corepressors found at the C terminus domain as well as CtBP, which may rescue the defect caused by the alteration of the dimerization module (13).

We extended our study of corepressor interactions to the family members of Ikaros. Like Ikaros, its three family members can bind all the Ikaros-interacting corepressors that we have tested (Sin3A, Sin3B, Mi-2, and Class I HDACs), with the exception of CtBP. CtBP, was recently shown to be capable of binding the Class II HDACs (28), and we have found that the Class II deacetylases, HDACs 5 and 6, can bind Ikaros. Class II HDACs can also bind Ikaros family members, but because they do not interact with CtBP (23), it is likely that the HDACs are recruited to them through CtBP-independent mechanisms. Given both the strong homologies between Ikaros family members and the conservation of most corepressor interactions, we expect that the findings with Ikaros may be extended to its family. Indeed, both Aiolos and Eos have been shown to be capable of repressing transcription (13, 27).

Several reports have indicated that aberrant repression may

FIG. 4. Transcriptional repression by Ik6 and is corepressor interaction mutants. NIH3T3 cells were transfected with 1 μg of the indicated Gal4 constructs (BXG1), the reporter G5tkCAT (10 μg), and the transfection efficiency control plasmid, pXGH5 (0.5 μg). Fold repression was calculated as described in Fig. 1A. The data represents the average of duplicate transfections repeated four times. Variation between experiments was less than 20%. A schematic diagram of Ik6 and its variants is presented; the filled square in the figure serves to depict the DNA binding domain of Gal4. Vertical rectangles indicates the presence of the two C-terminal zinc fingers. The absence of this notation indicates a mutation of DL to AS in the CtBP interaction motif of Ik6 that prevents its interaction with this corepressor (23). Wherever a deletion has been made the amino acids deleted are indicated (dl).
Fig. 5. Conservation of corepressor interactions across Ikaros family members. A, 293T cells were co-transfected with 10 μg each of an expression vector for MT-Sin3A and the FLAG-tagged Ikaros family members, Aiolos (FLAG-A), Helios (FLAG-H), or Eos (FLAG-E). IP were performed on whole cell lysates with antibodies to the FLAG epitope. IB analysis with antibodies to the Myc epitope (MT) revealed interactions between the Ikaros family members and Sin3A. Blots were stripped and reprobed with the antibody used for the IP to ascertain that the epitope-tagged proteins were indeed immunoprecipitated. B, interactions between HA-tagged Sin3B (long form) and Aiolos, Helios, and Eos were tested as in A. Immunoblot analysis was performed using HA antibody to identify interactions between Sin3B (long form) and the Ikaros family members. C, interactions between endogenous Mi-2 and Ikaros family members were tested as in A. D, interactions between endogenous HDAC2 and Ikaros family members were tested as in A. E, comparison of Class II HDAC interactions with Ikaros family members. 293T cells were transfected with Myc-tagged HDAC4 (MT-HD4), HA-tagged HDAC5 (HA-HD5), and HDAC7 (HA-HD7) and either FLAG-tagged Ikaros, Aiolos, Eos, or Helios. Interactions between the Ikaros family members and these Class II HDACs were determined by IP. The numbers below the blot are intended to aid the reader in correlating the input and IP lanes. F, comparison of Class I (HD1 and 3) versus Class II (HD4, 5, and 7) HDAC interactions with Ik1. 293T cells were transfected with FLAG-tagged Ik1 together with either FLAG-tagged HDAC1 (FlagHD1), FLAG-HDAC3 (FlagHD3), Myc-tagged HDAC4 (MT-HD4), HA-tagged HDAC5 (HA-HD5), or HDAC7 (HA-HD7). Interactions between the Ikaros family members and these Class II HDACs were determined by IP with the indicated antibodies. The numbers below the IB are intended to aid the reader in identifying the input and IP lanes.
play an important role in carcinogenesis (29). In this context, it is noteworthy that mice expressing dominant-negative Ikaros proteins (e.g. Ik6) develop leukemias and lymphomas with complete penetrance (10). It has been argued that these cancers may arise because of the cellular anomalies that result from the titration of DNA-binding Ikaros family proteins from their binding sites by the dominant-negative proteins (1). In addition, it is also possible that the large-scale incorporation of Ik6 into Ikaros complexes may alter their transcriptional function, which could severely impact lymphocyte homeostasis. The identification of a repression-defective dominant-negative Ikaros protein may permit a distinction between the role of aberrant repression versus that of titration of Ikaros family members in dominant-negative Ikaros-mediated lymphomagenesis.

In conclusion, in this report we have (a) identified the minimal repression modules and corepressor interaction interfaces on Ikaros, (b) uncovered redundancies in corepressor interaction modules involved in Ikaros-mediated repression, (c) constructed Ikaros proteins defective for interaction with the majority of the corepressors of Ikaros, and (d) shown the importance of Mi-2β, HDAC2, and CtBP in Ikaros-mediated repression. This provides a useful starting point for future studies aimed at understanding the molecular mechanisms by which Ikaros represses gene expression.

Acknowledgments—We thank Dr. B. Morgan for his gift of the Flag-Eos plasmid and Drs. E. Seto, M. Lazar, and R. Evans for their gift of the plasmids encoding Class I and II histone deacetylases.

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