CREM-Dependent Transcription in Male Germ Cells Controlled by a Kinesin

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ACT is a LIM-only protein expressed exclusively in round spermatids, where it cooperates with transcriptional activator CREM in regulating various postmeiotic genes. Targeted inactivation of CREM leads to a complete block of mouse spermiogenesis. We sought to identify the regulatory steps controlling the functional interplay between CREM and ACT. We found that ACT selectively associates with KIF17b, a kinesin highly expressed in male germ cells. The ACT-KIF17b interaction is restricted to specific stages of spermatogenesis and directly determines the intracellular localization of ACT. Sensitivity to leptomycin B indicates that KIF17b can be actively exported from the nucleus through the Crm1 receptor. Thus, a kinesin directly controls the activity of a transcriptional coactivator by a tight regulation of its intracellular localization.

In contrast to somatic cells, transcriptional activation by CREM in male germ cells is CBP- and phosphorylation-independent (*1*, *2*). CREM is essential for spermatogenesis because its mutation in the mouse results in early block of spermiogenesis (*3*, *4*). A number of studies have placed CREM into a pivotal position as a master regulator of several postmeiotic genes. This central function is obtained through the physical and functional association between the activation domain of CREM and ACT (activator of CREM in testis), a LIM-only protein with coactivator properties (*2*). ACT belongs to a family of proteins that share a characteristic organization of four and a half LIM domains (*5*). LIM domains are composed of two adjacent zinc fingers involved in protein-protein interactions (*6*). This unique CREM-ACT activation mechanism in male germ cells prompted us to further elucidate the regulatory pathways implicated by searching for proteins interacting with ACT. A yeast two-hybrid screen was set up using a murine testis cDNA library with full-length ACT as a target protein. In this assay, ACT, fused to the GAL4 DNA binding domain, displays very low activity (fig. S1A). In tests for nutritional selection and β-galactosidase activity, one clone was found a large number of times and showed high affinity for ACT (fig. S1, A and B).

Unexpectedly, the clone corresponded to an internal sequence of a kinesin protein. The full-length sequence (fig. S1C) revealed an isoform of the brain-specific KIF17 (*7*). After the proposed kinesin nomenclature (*8*), we termed this protein KIF17b. The *Kif17b* gene encodes a protein of 1039 amino acids, one residue more than KIF17, and shows a few differences in the sequence as compared with KIF17 (fig. S1C).

The kinesin superfamily proteins (KIFs) have been shown to transport organelles, vesicles, protein complexes, and RNA to specific destinations in a microtubule- and adenosine triphosphate–dependent manner (*9*, *10*). In addition, some KIFs participate in chromosomal and spindle movements during mitosis and meiosis (*11*). Our finding suggests that a kinesin may be directly involved in regulating the function of a transcriptional coactivator.

Kif17b is expressed at high levels exclusively in testis (Fig. 1A). We analyzed the developmental pattern of *Kif17b* expression. In situ studies show that expression is restricted to the inner rim of seminiferous tubuli, a location corresponding to postmeiotic cells (Fig. 1D). In the developing testis, *Kif17b* expression is detected from the age of 3 weeks onward (Fig. 1B), a time when round spermatids appear, strictly paralleling ACT expression (*2*). An antibody to KIF17 (*7*), which also recognizes KIF17b, reveals a protein of the expected size (relative molecular mass of about 170 kD) in purified spermatids that coexpress ACT (Fig. 1C). Thus, KIF17b and ACT display an overlapping pattern of developmental expression.

KIF17b and ACT show a strong association in vitro and in vivo. In the two-hybrid assay, KIF17b and ACT display about the same efficacy of interaction as ACT and CREM (fig. S2A). The two proteins associate very efficiently also in a glutathione *S*-transferase–pull down assay and by coimmunoprecipitation after coexpression in transfected cells (fig. S2, B and C). Finally, and most important, coimmunoprecipitation of native proteins from a mouse testis total extract confirms the ACT-KIF17b association in

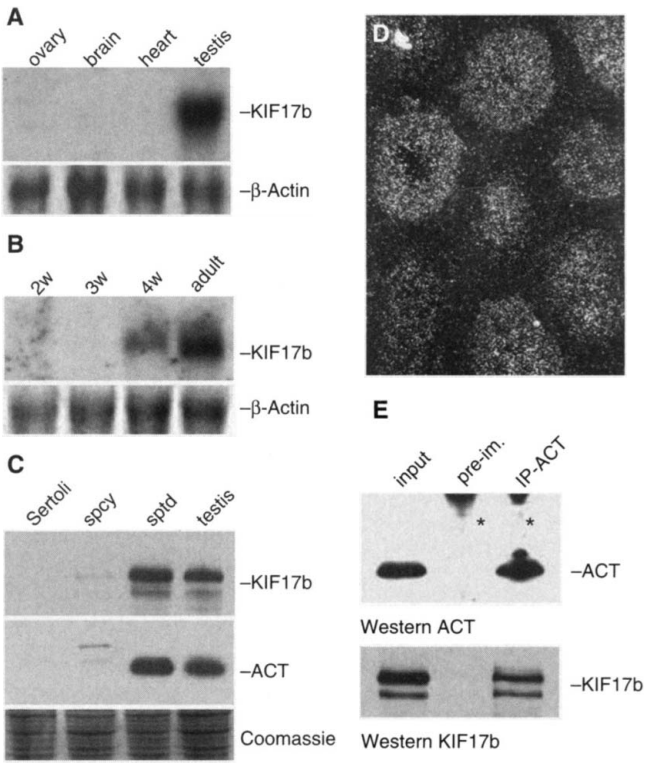

Fig. 1. Analysis of *Kif17b* expression. (**A**) *Kif17b* is testis-specific. Total RNA (10 μg) from mouse tissues analyzed by Northern blot with a *Kif17b*-specific probe (internal fragment amino acid residues +958 to +2160). (**B**) Northern analysis of 10 μg of testis RNA from mice of different ages. (**C**) Western analysis using ACT- and KIF17-specific antibodies with whole-cell extracts from purified Sertoli cells, spermatocytes (spcy), spermatids (sptd), and testis. (**D**) In situ hybridization analysis of *Kif17b* expression in mouse testis. (**E**) Association of native ACT and KIF17b proteins. Coimmunoprecipitation (IP) of KIF17b-ACT from total testis extracts of 12-week-old mice using the ACT-specific antibody. Western analysis using the ACT- and KIF17-specific antibodies (*25*). Asterisks indicate heavy-chain immunoglobulins. pre-im. is pre-immune serum.

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vivo (Fig. 1E). Thus, the association between ACT and KIF17b is unequivocal.

In our previous studies ACT was found to be nuclear in round spermatids as determined by fluorescence microscopy on immunostainings of mouse testis sections (*2*). However, most kinesin proteins not involved in mitosis are described as cytoplasmic proteins (*9*, *10*). We thus revisited the localization of ACT at all stages of spermatid development (*12*) by squash preparations of defined stages of the seminiferous tubule (*13*), coupled to confocal microscopy. This method is highly sensitive. We confirmed that ACT is nuclear in round spermatids (Fig. 2),[5] going from a weak expression in stage I to a strong expression in stage VI. However, starting from stage VIII, some cytoplasmic expression becomes apparent. During spermatid elongation (from stage IX onward), cytoplasmic expression becomes more dominant and coincides with a depletion of ACT expression from the nucleus. In stage XI expression disappears. Parallel samples analyzed for KIF17b expression reveal nuclear and cytoplasmic expression in round spermatids. In stage VIII a dual expression pattern of KIF17b can be observed. The pattern is nuclear and cytoplasmic in some spermatids but only cytoplasmic in others, indicating a possible shuttling activity of this protein. During spermatid elongation KIF17b and ACT expression coincide, and KIF17b becomes depleted from the nucleus. KIF17b expression remains on the nuclear border until stage XII. Thus, the two proteins display a coupled intracellular localization in male germ cells.

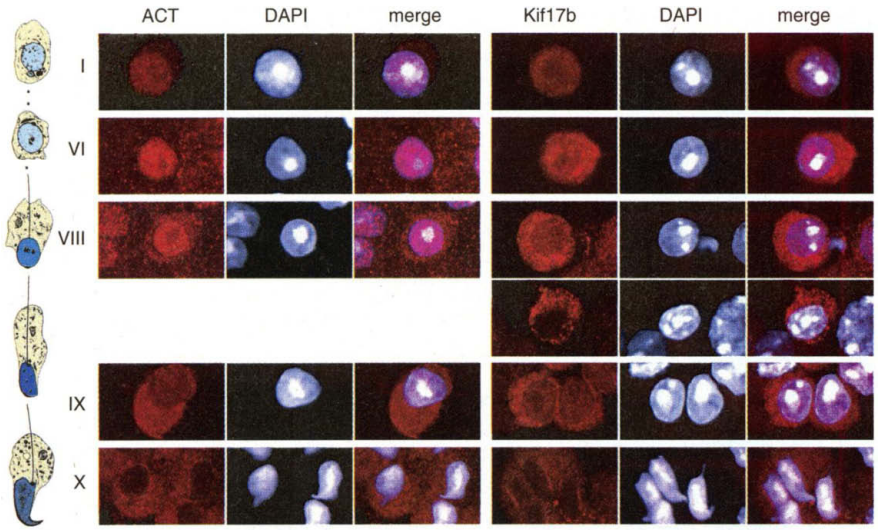
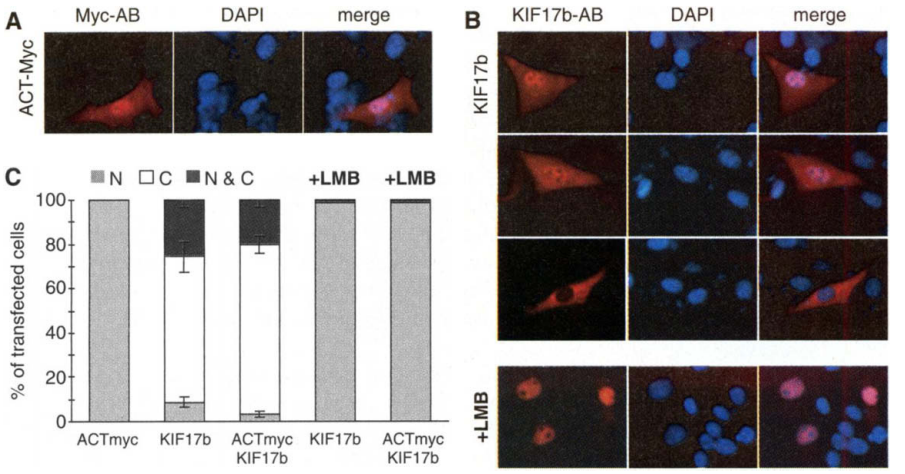


Fig. 2. Nucleo-cytoplasmic movements of KIF17b and ACT in mouse spermatids. Squash preparations from segments of defined stages of the mouse seminiferous tubule (*12*) in sequential order were obtained by transillumination-assisted microdissection (*13*). Parallel samples were fixed and used for immunocytochemical analysis with the ACT- and KIF17-specific antibodies and analyzed with confocal microscopy (*25*). Schematic representation of the developing spermatid during the stages of spermatogenesis (*12*) is shown on the left.



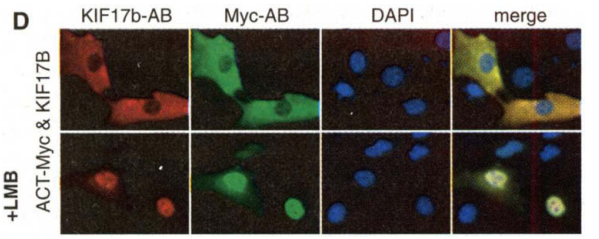


Fig. 3. Colocalization of KIF17b and ACT in transfected COS and NIH3T3 cells. Monoclonal antibody Myc9E12 and the KIF17b-specific antibody were used (*25*). (**A**) Myc-ACT alone is mainly nuclear. (**B**) KIF17b alone is differentially expressed in cytoplasm (majority of cells), nucleus and cytoplasm, or mainly nucleus; upon inhibition of Crm1-dependent nuclear export with leptomycin B (LMB), KIF17b is being sequestered in the nucleus (bottom panel). (**C**) Cell counts for histograms were obtained from three independent transfections (mean ±SD). (**D**) ACT and KIF17b together display strict colocalization with a distribution pattern similar to that of KIF17b alone; LMB treatment sequesters the Myc-ACT-KIF17b complex in the nucleus (bottom panel).

Treatment of transfected cells with leptomycin B (LMB), a specific inhibitor of nuclear export mediated by leucine-rich nuclear export signals (NESs) (*14*, *15*), results in localization of KIF17b to the nucleus (Fig. 3B, bottom panel). Myc-ACT localization is unaltered by LMB treatment, whereas the Myc-ACT-KIF17b complex is totally sequestered in the nucleus (Fig. 3D, bottom panel). Sensitivity to LMB indicates that KIF17b can be actively exported from the nucleus to the cytoplasm by NES-mediated nuclear export through the Crm1 receptor (*16*–*18*).

Through its potential to bind ACT and determine its exclusion from the nucleus, overexpression of KIF17b should inhibit ACT-dependent transcriptional activation (*2*). We tested the promoters of the calspermin and the angiotensin-converting enzyme (ACE) genes, two physiologically relevant targets whose activation was previously demonstrated to be CREM dependent in male germ cells (*2*). A dose-dependent decrease of the ACT-dependent transcriptional activation

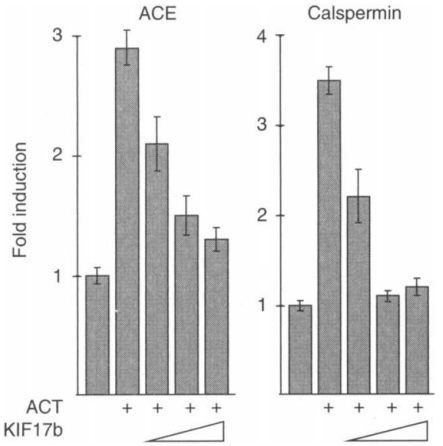


Fig. 4. KIF17b represses ACT-dependent transcriptional activation. COS cells transfected with 0.5 μg of ACE-CAT or calspermin-CAT (*2*), 2 μg of CREM and 3 μg ACT with increasing amounts of pSG5-Kif17b. Activation is reported as fold induction of CREM-induced promoter. Transfection efficiency was monitored by β-galactosidase assays using the CMVβ-gal plasmid. Data are means ±SD of results from three independent transfection experiments (*25*).

by KIF17b was observed (Fig. 4).[6] This effect is specific, as confirmed by the use of an unrelated kinesin and another transcriptional activator (VP16). It was also observed on a Gal4-based heterologous promoter (fig. S3). Sequestering ACT-KIF17b into the nucleus by LMB treatment leads to recovery of ACT-dependent transcriptional activation (fig. S3).

The findings presented here expand the view of the role that kinesins exert in cellular physiology. We have linked one kinesin directly to a transcriptional coactivator, while examples of indirect regulations were previously reported (*19*, *20*). The transduction events that govern KIF17b to directly modulate ACT-mediated transcription have yet to be explored, but another LIM-only protein with transcriptional coactivator properties, FHL2, is able to transmit Rho signals from the cell membrane to the nucleus (*21*). In addition, a mitotic kinesin-like protein was recently found to be intimately connected to Rho-dependent signaling and to be required for microtubule bundling (*22*). Growing evidence points to a functional connection between microtubule cytoskeleton integrity and transcriptional activation, as shown in the cases of factor MIZ-1 activated in response to sterols (*23*), and *Costal2*, an inhibitor of cubitus interruptus in *Drosophila* (*24*).

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Supporting Online Material

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Materials and Methods
Figs. S1 to S3
References

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Sir2-Dependent Activation of Acetyl-CoA Synthetase by Deacetylation of Active Lysine

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Acetyl-coenzyme A (CoA) synthetase (Acs) is an enzyme central to metabolism in prokaryotes and eukaryotes. Acs synthesizes acetyl CoA from acetate, adenosine triphosphate, and CoA through an acetyl–adenosine monophosphate (AMP) intermediate. Immunoblotting and mass spectrometry analysis showed that *Salmonella enterica* Acs enzyme activity is posttranslationally regulated by acetylation of lysine-609. Acetylation blocks synthesis of the adenylate intermediate but does not affect the thioester-forming activity of the enzyme. Activation of the acetylated enzyme requires the nicotinamide adenine dinucleotide–dependent protein deacetylase activity of the CobB Sir2 protein from *S. enterica*. We propose that acetylation modulates the activity of all the AMP-forming family of enzymes, including nonribosomal peptide synthetases, luciferase, and aryl- and acyl-CoA synthetases. These findings extend our knowledge of the roles of Sir2 proteins in gene silencing, chromosome stability, and cell aging and imply that lysine acetylation is a common regulatory mechanism in eukaryotes and prokaryotes.

Members of the Sir2 family of proteins (sirtuins) are nicotinamide adenine dinucleotide (NAD^+)–dependent deacetylase enzymes involved in chromosome stability, gene silencing, and cell aging in eukaryotes and archaea (*1–3*). All previously known substrates of sirtuins are components of chromatin and/or affect gene transcription. Strains of the enterobacterium *S. enterica* lacking sirtuin (encoded by the *cobB* gene) cannot grow on propionate and in low acetate concentration as carbon and energy sources because the acyl-CoA synthetases responsible for converting free acids into acyl-CoA derivatives are inactive (*4*, *5*). Our work shows that acetyl-CoA synthetase activity (Fig. 1A) (encoded by the *acs* gene) is regulated by posttranslational acetylation. We also show that activation of acetylated Acs requires CobB deacetylase activity.

Acs enzyme synthesized by a $cobB^-$ strain of *S. enterica* was inactive in crude cell-free extract (*5*). To address the possibility that Acs activity was posttranslationally regulated, we overexpressed the *acs* gene of *S. enterica* in $cobB^+$ and $cobB^-$ strains and purified the protein as described in (*6*). Acs

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