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Characterisation of a novel Kinesin Light Chain

by

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ABSTRACT

Here we characterize a novel Kinesin Light Chain (KLC) gene, KLC-3. The novel gene is classified as a KLC based on sequence comparisons to known KLC genes and its interaction with Kinesin Heavy Chain and microtubules *in vitro*. Subsequently, we analyze KLC and KHC gene expression in the testis by RT-PCR, Western and immunolocalisation. The RT-PCR and Western analysis demonstrate that KLC-3 expression is highest in spermatids, and that it is the sole KLC expressed in these cells. Of the KHC genes, only Kif5c is expressed in these cells, however at relatively low levels. Furthermore, KLC-3 localizes to sperm tails, whereas Kif5c does not. This suggests either a novel, non-KHC related function for KLC-3, or the presence of a new KHC gene in sperm tails. Based on yeast two hybrid experiments, which demonstrate interaction with structural sperm tail proteins that do not co-localize with microtubules, we favor the former hypothesis.

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LIST OF ABBREVIATIONS

ERCC-2 – Excision Repair Cross Complementing rodent group 2 gene
HR- Heptad Repeat Region of KLCs
KLC – Kinesin Light Chain
KHC – Kinesin Heavy Chain
Kif5a,b,c – isoforms of KHC in higher eukaryotes
MBO – Membrane Bound Organelles
MBP – Maltose Binding Protein
PEST – Protease sensitive Region of KLCs
TPR – Tetra trico Peptide Repeats, a protein motif in KLCs

SUMMARY OF INTRODUCTION, RESULTS AND DISCUSSION

Kinesin is a motor molecule for the transport of cytoplasmic organelles. It is composed of two heavy chains, which generate motor function, and two light chains. Proposed functions for the light chains include mediating the binding of cargo, regulating motor function, and/or as a target for regulatory signaling cascades. Since Kinesin Light Chain (KLC) independent mechanisms have been suggested, a reasonable hypothesis is that KLCs introduce diversity to these functions. Alternative functions are also possible, as traditional paradigms of kinesin function -based largely on neuronal systems, are being challenged by recent observations of non-motor based kinesin functions in different model systems. The testis is a good in vivo model system for the study of protein function in differentiation, and thus potentially unique functions of different kinesin isoforms in various cell types. Furthermore, the presence of multiple and unusual microtubule structures makes it an attractive tissue for the study of potentially atypical kinesin functions.

In this report, we expand upon previous research from our laboratory on a putative Kinesin Light Chain, KLC-3. The gene had previously been cloned by virtue of its interaction with Mos and shown to complex with Mos *in vivo*. KLC-3 has also been shown to localize to the midpiece of elongating spermatid tails and associate primarily with Outer Dense Fibers and mitochondria. Based on a PCR strategy it is shown that KLC-3 is identical to a previously identified but putative KLC gene in the ERCC2 locus. Sequence analysis and protein alignments demonstrate that KLC-3 has high homology to other KLCs in the signature KLC motifs. Additionally, KLC-3 co-immunoprecipitates

with KHC *in vitro*, and deletion of the putative KHC interacting domain abolishes this interaction. Furthermore, it interacts with microtubules in a nucleotide dependent manner. Based on these *in vitro* characteristics, it is concluded that KLC-3 is indeed a novel member of the KLC family.

Subsequent analysis of kinesin expression during sperm differentiation by RT - PCR suggested that expression of KLC genes differs by cell type, and that KLC-3 appears to be in excess of KHC in spermatids. In confirmation of this, Western analysis and immunofluorescence failed to detect any KHC in spermatid tails, and further confirmed previous localization of KLC-3 to this structure. It is concluded that KLC-3 is the only known KLC expressed in elongating spermatids, and that the large majority of it likely does not interact with any known KHC, but rather may engage in a novel and undefined function. These results also support concepts of sub-specialization of kinesin genes. Additionally, it is shown that KLC-3 interacts with sperm tail proteins ODF-1 and SPAG4 in the yeast two hybrid system, and that the respective leucine zippers may mediate this interaction. This suggests possible mechanisms for targeting KLC-3 to the midpiece of the sperm flagellum. Finally, in yeast KLC-3 does not interact with the kinase domain of c-Mos, but rather with an N-terminal region. This suggests that KLC-3 is not a regulatory target for Mos signaling. The significance of this interaction in the testis is not clear, but in other tissues could explain observations of Mos localization to microtubules. The implications and shortcomings of these experiments are discussed.

INTRODUCTION

I: OVERVIEW

I.a: The Cytoskeleton

The cellular cytoskeleton is a vital component of all eukaryotic cells, and is responsible for a wide range of functions including the maintenance of cellular integrity, organelle transport, the development of cell polarity and extensions, cellular motility, meiotic and mitotic movements, and membrane trafficking. The cytoskeleton is composed of microtubules, actin filaments, and intermediate filaments, with distinct but oft overlapping functions, which are the subject of much current investigation. Microtubules (MTs) play a role in many of the functions described above. Microtubules are composed of 13 individual protofilaments, each composed of repeating tubulin dimers. Microtubules are nucleated at the Microtubule Organizing Center (MTOC), which is often perinuclear in location. The microtubule is polarized in the sense that the end anchored at the MTOC (minus end) is static relative to the generally rapidly growing/shrinking plus end, which frequently projects to the cell's peripheries. Growth and reduction of MTs is achieved by simple addition of tubulin dimers, mostly at the plus end. The function and dynamic nature of microtubules is partially regulated and determined by an extended family of molecular motor proteins. Kinesin and dynein are the prototypes for this family of motors. The conventional view is that these motors bind to both microtubules and molecular cargo such as vesicles, and hydrolyze ATP to achieve motion along the MT "tracks", thereby transporting cargo within the cell. More recently, motors have also been implicated in regulating the dynamics of MT assembly. The

importance of intracellular movements is put into context when one realizes that all cellular movement of large particles, and/or over large distances, and/or at regulated moments cannot be effected by diffusion alone. Together with the analogous actin/myosin system, the cytoskeleton appears to be solely responsible for such movement. Baltimore, 1990, offers a comprehensive introduction to the composition and function of the cytoskeleton, and (Drubin and Hirokawa, 1998) presents a compilation of recent advances in this field for interested readers.

I.b: Kinesin

Conventional kinesin (referred to throughout as kinesin) is a plus-end directed, microtubule based molecular motor (Vale et al., 1985a; Vale et al., 1985b; Brady, 1985). It is a mechanochemical enzyme, which powers the cytoplasmic transport of organelles and Membrane Bound Vesicles (MBOs) on microtubules reviewed in (Brady, 1991). Kinesin is a tetramer of two heavy chains (KHC) and two light chains (KLC) (Bloom et al., 1988; Johnson et al., 1990; Kuznetsov et al., 1988). KHC has a tripartite structure (Yang et al., 1989) consisting of distinct domains with defined functions. At the N-terminus is a globular head which hydrolyzes ATP, binds microtubules and is responsible for translocation on microtubules (Bloom et al., 1988; Hirokawa et al., 1989) (Penningroth et al., 1987; Scholey et al., 1989; Yang et al., 1990; Kuznetsov et al., 1989). The middle to C-terminal segment of KHC consists of an alpha-helical, coiled-coil rod-like region which mediates dimerisation (de Cuevas et al., 1992). The extreme C-terminus of KHC consists of a small globular domain which is the site of KLC and putative cargo attachment (Hirokawa et al., 1989). The role of the light chains is not as

clearly defined as that of the heavy chains, as they appear to be dispensable for in vitro motility (Kuznetsov et al., 1989;Yang et al., 1990)and in vitro binding to membrane bound vesicles (Skoufias et al., 1994). Subsequent to the cloning of KHC, a large and ever expanding family of Kinesin Related Proteins (KRP) has been identified (Nakagawa et al., 1997). The family is characterized by a highly conserved motor domain for microtubule-based transport. Like kinesin, the KRPs are thought to mediate transport of MBOs, and additionally have been implicated as key motors in mitotic and meiotic chromosome and spindle dynamics, the control of microtubule dynamics, transport of soluble protein components on recyclable "rafts", RNA movements, and potentially in functions not involving MT based motility. Unlike kinesin, the KRPs do not have associated light chains, although they do associate with various (often unidentified) Kinesin Associated Proteins (KAPs). For reviews see (Endow, 1991; Hirokawa et al., 1998; Goldstein and Philp, 1999). Kinesin has been extensively characterized biochemically, genetically and at the atomic level. With well over a 1000 publications on kinesin and KRPs, the scope of these investigations precludes a comprehensive discussion here. The following reviews are recommended: comprehensive (Bloom and Endow, 1995; Bloom and Endow, 1994; Goldstein and Philp, 1999), atomic structure and force generation (Block, 1998), motors in membrane trafficking and the extended kinesin and dynein family (Hirokawa et al., 1998), and a recent review (Vale and Milligan, 2000). Another excellent source of information is the kinesin home page (www. www.scripps.edu/~wriggers/projects/kinesin/).

I.c: Kinesin Light Chains

The study of KLCs lags behind that of KHC or KRPs. KLCs were originally suggested to mediate either regulation of kinesin (Hollenbeck, 1993) or cargo binding to kinesin (Hirokawa et al., 1989). The first KLCs were cloned from rat brain and shown to exist as splice variants (KLC a, b and c) (Cyr et al., 1991). This variance was suggested to allow the generation of functional diversity. Analysis of the sequence and hypothetical protein structure suggested putative binding sites for KHC and molecular cargo. Subsequently, homologues of rat KLC were cloned from multiple other organisms, with similar predictions made(Beushausen et al., 1993; Cabeza-Arvelaiz et al., 1993; Fan and Amos, 1994; Gauger and Goldstein, 1993; Wedaman et al., 1993). In addition to existing as splice variants, KLCs are also a multi gene family (KLC-1 and 2)(Lamerdin et al., 1996; Rahman et al., 1998). Recent reports have shown the predictions of KLC function and structure may be largely correct (Diefenbach et al., 1998; Gindhart, Jr. et al., 1998; Khodjakov et al., 1998; Lindesmith et al., 1997a; Rahman et al., 1999; Stenoien and Brady, 1997; Verhey et al., 1998). However, the precise function of KLCs remains a matter of controversy. These investigations and their implications in the context of the general kinesin field are discussed below.

II. REVIEW OF PUBLISHED DATA ON KINESIN LIGHT CHAINS

II.a: Sequence and Structure of KLCs

Some of the initial insights into KLC function came from an analysis of their sequences and the ensuing predicted protein motifs. KLCs were first cloned from rat brain, and subsequently, homologues were cloned from squid, *C.elegans*, *Drosophila*, human, mouse, sea urchin and hamster (Cyr et al., 1991; Beushausen et al., 1993;

Cabeza-Arvelaiz et al., 1993; Fan and Amos, 1994; Gauger and Goldstein, 1993; Rahman et al., 1998; Wedaman et al., 1993) (Khodjakov et al., 1998). To ease confusion with the current nomenclature, all the homologues of rat brain KLC (inclusive) will be referred to as (organism)KLC-1. A new KLC gene has been identified from mouse (KLC-2) (Rahman et al., 1998), and sequencing of the ERCC-2 locus revealed the potential existence of a potential third KLC gene, KLC-3 (Lamerdin et al., 1996). Analysis of the cloned sequences, coupled with deletion analysis, co-transfections and the use of various other techniques by these groups has revealed that KLCs share some highly conserved sequence motifs, likely with specific functions: (from N to C terminus)

i) Multiple start methionines, likely to generate alternate isoforms.

ii) The conserved Heptad Repeat (HR) region, which is predicted to fold as a coiled-coil, and is thought to mediate binding to KHC (Diefenbach et al., 1998; Gauger and Goldstein, 1993; Verhey et al., 1998).

iii) A PEST region, which has low sequence conservation, but with a large percentage of amino acids represented by proline, serine, threonine and aspartate, and which is vulnerable to proteolytic cleavage (Beushausen et al., 1993).

iv) A region of six long, imperfect tandem repeats resembling tetra-trico peptide repeats (TPR)(Gindhart, Jr. and Goldstein, 1996). This motif is the most highly conserved region of the KLCs, and as such has been suggested to lend KLCs their characteristic fan-shape(Cyr et al., 1991). The tetra-trico repeats are predicted to form amphipathic helices, and in other proteins are known to mediate protein-protein interactions (reviewed in Lamb et al., 1995). KLCs have six such repeats, and interestingly the individual repeats are highly conserved amongst species - more so than individual

repeats from the same organism. Eight amino acids are loosely conserved in the TPR motifs of other proteins (Lamb et al., 1995), and other KLC family members conserve approximately 5 of these 8 in the individual TPR motifs. The TPR region has been shown to mediate binding of kinesin to membrane bound vesicles (Stenoien and Brady, 1997).

v) Completely divergent sequences at the extreme N-terminus and the C-terminus, which is additionally subject to alternative splicing mechanisms. (Cyr et al., 1991). Splicing has been shown to generate functional diversity among KLC isoforms: splice isoform specific C-terminal sequences likely target the isoforms to particular molecular cargoes (Khodjakov et al., 1998; Gyoeva et al., 2000).

vi) A number of putative phosphorylation sites throughout the molecule, including PKA, PKC and tyrosine kinase sites (Beushausen et al., 1993).

II.b: Expression Patterns, Subcellular Localisation and Gene Targetting Experiments suggest Functional Diversity of KLCs

i) Expression of Kinesin in Eukaryotes

An examination of the expression patterns and subcellular localization of the KLCs gives additional clues to their roles within the cell. Kinesin and KRPs appear to be expressed in virtually all eukaryotes. Their widespread expression suggests that they play an essential role in cell physiology. KHC homologues have been cloned from several fungal species, including *Ustilago maydis* (Lehmler et al., 1997), *Neurospora crassa* (Steinberg and Schliwa, 1995), *Syncephalum racemosum* (Steinberg, 1997), and *Schizosaccharomyces pombe* (Brazer et al., 2000). Furthermore kinesin family proteins have also been isolated from plants (Mitsui et al., 1993). However, despite the fact that

the fungal kinesins appear to have a similar function to their counterparts in higher eukaryotes (reviewed in Steinberg, 2000), these fungi do not express associated light chains, and no light chains have been cloned from plants. Finally, recall that the carboxyl terminus of KHC from higher eukaryotes is capable of binding membrane bound vesicles in the absence of associated light chains (Skoufias et al., 1994). These findings suggest that KHC is functional by itself, and question the necessity/function of the light chains.

If we consider that KLCs are hypothesized to regulate kinesin activity and mediate cargo binding, then the evolution of (multiple) KLC genes in higher eukaryotes presents an attractive hypothesis: the increased complexity of higher eukaryotes requires additional specialization of kinesin activity and targeting, which is achieved by means of diversification of the kinesin isoforms. A parallel between animal complexity and the number of kinesin genes - both light chains and heavy chains, supports this argument. *Drosophila*, *C. elegans*, squid and sea urchin are thought to encode a single KLC and KHC gene, mice and humans on the other hand are known to encode at least three of each: KLC-1, 2 and 3 (Rahman et al., 1998; Lamerdin et al., 1996), and Kif5a, b, and c (Navone et al., 1992; Niclas et al., 1994; Xia et al., 1998; Nakagawa et al., 1997). While the fungi have only one potential kinesin complex, mammals have three KHCs and KLCs -each capable of forming a kinesin complex with the other, as it appears that various combinations of light chain and heavy chain are viable (Rahman et al., 1998). Additional diversity can be generated by the various splice isoforms of KLCs, thus generating - theoretically- a large number of kinesin complexes, each potentially unique in its cellular function. Likewise, a similar correlation in the number of KRPs with animal complexity

has become evident from studies on published genomes (reviewed in Goldstein and Philp, 1999).

ii) Tissue and Cellular Expression Patterns

The hypothesis of kinesin isoform specialization is supported by an examination of the cellular expression patterns of the various KHC and KLC isoforms. Initial claims that mKLC-1 is mostly neuronal and mKLC-2 is ubiquitous (Rahman et al., 1998) are likely only partially accurate. Examination of the relevant figures shows that KLC-1 is expressed in most tissues. This is supported by reports which detect chicken KLC-1 in virtually all its tissues (Hollenbeck, 1989a) and numerous human cell lines and various human tissues (Cabeza-Arvelaiz et al., 1993; Stenoien and Brady, 1997; Khodjakov et al., 1998), albeit at varying levels. However, the ratio of mKLC-1 to mKLC-2 expression certainly does differ amongst tissues: mKLC-1 is very prominent in brain and mKLC-2 is more prominent in other tissues (Rahman et al., 1998). This suggests unique needs for specific KLC genes in particular tissues. Furthermore, mKLC-1 and 2 expression does differ by cell type in the mouse brain: mKLC-1 for example is not expressed in Schwann cells, whereas mKLC-2 is. Also the subcellular immunofluorescence patterns of the two genes differ from each other in neuronal cells, suggesting differences in putative cargos (Rahman et al., 1999). Thus, the expression of individual KLC genes is unique and may reflect a need for functional diversity.

At least in other organisms, KLC-1 splice isoforms also differ in their expression patterns: in squid, splice isoform abundance and expression was found to differ by tissue (Beushausen et al., 1993), and in the rat brain, rKLC-1 a,b and c isoforms were found to be expressed in different regions of the brain (Su et al., 1997). Furthermore, recent

reports demonstrate that different splice isoforms of hamster KLC-1, differing only in their C-terminal sequences, localize to specifically to distinct organelles within the same cell type (Khodjakov et al., 1998).

Similar results have been obtained in experiments delineating KHC isoform expression. Kif5b is a ubiquitous kinesin, although its expression level is known to vary amongst the tissues (Hollenbeck, 1989a). Kif 5a on the other hand is expressed exclusively in neuronal tissues (Niclas et al., 1994), and its expression differs from that of Kif5b during neuronal development (Vignali et al., 1996; Vignali et al., 1997). Kif 5c is also thought to be neuronally enriched. As with the KLC genes, the tissue specific expression pattern of the different Kif isoforms has been interpreted as signifying distinct or specialized roles for the various kinesins in these tissues. In support of distinct roles for the individual KHC genes, there is evidence that molecular weight variants of KHC mediate transport of different membrane bound organelles in rat axons. It is unclear from these studies whether these variants correspond to different genes or splice isoforms (Elluru et al., 1995). This theme is also consistent with current views on the functions of individual members of the extensive family of Kinesin Related Proteins (reviewed in Hirokawa, 1998).

Collectively, these results provide strong evidence that the individual KLC and KHC genes and isoforms differ in their specific cellular function. Therefore, the increased diversity of kinesin molecules in higher organisms could well be a consequence of their increased cellular specialization and diversification, and a concomitant need for multi-facetted cargo transport.

iii) Gene Targetting Experiments

An alternate to the hypothesis presented above is that multiple kinesin genes are present in higher eukaryotes simply as a mechanism of redundancy to avoid lethality in the case of mutation. Certainly, redundancy has been implicated as a factor for the KRP family, whose functions overlap with that of kinesin (reviewed in Goldstein and Philp, 1999). The results of KHC gene targeting experiments partially support the view of functional redundancy. In mice, Kif5b knockouts prove to be embryonic lethal (Tanaka et al., 1998), as is the case for *Drosophila* KHC mutants (Saxton et al., 1991). Recall that mice encode three KHC genes, whereas *Drosphila* encodes only a single gene. Collectively, this indicates that neither Kif5a nor c is able to compensate for the absence of Kif5b in the mouse, and therefore these KHC genes are not functionally redundant but rather specialized for a particular function. However, the possibility of redundancy is not ruled out by these experiments. For example, in *Drosophila* the lethality appeared to be a consequence of a neuronal defect (Hurd and Saxton, 1996), and in mouse it was possible to culture extra embryonic cells (Tanaka et al., 1998), suggesting that at least in some cell types KHC is either not required or redundant.

KLC gene targeting experiments in various organisms suggest that KLCs are required for proper kinesin function in organisms, which encode them. In *Drosophila* for example, the loss of KLC function results in a lethal phenotype very similar to that of the KHC mutants (Gindhart, Jr. et al., 1998), suggesting that KLC is absolutely required for proper kinesin function. Unfortunately, neither the localization nor the activity/biochemical properties (ie. ATPase, etc) of KHC were analyzed in the mutant *Drosophila* embryos. Mice KLC knockouts are viable but also exhibit a phenotype,

demonstrating that the various KLC genes cannot compensate for one another: mKLC-1 knock-outs are smaller than their wild-type counterparts and exhibit neuromuscular defects, consistent with high mKLC-1 expression in neuronal tissues. Furthermore, there was no up regulation of mKLC-2, as would be expected if mechanisms ensuring redundancy existed (mKLC-3 was not analyzed) (Rahman et al., 1999). These results indicate that the mKLCs are not functionally redundant, but rather play different roles within the cell. Additionally, a subset of Kif5a and c was found to localize aberrantly in neuronal tissues, whereas Kif 5b localization was normal. This suggests that certain combinations of Kif and KLC are preferred in certain tissues, presumably since they fulfill particular functions here.

Further support comes from observations that native kinesin always exists as a tetramer in organisms which encode KLC genes, emphasizing the importance of light chains to proper kinesin function (the purification of KHC without associated light chains from higher eukaryotes is an experimental artifact (Bloom and Endow, 1994)). Thus, light chains appear to be essential for proper kinesin function, at least in *Drosophila* and mice. The KHC genes have likely evolved such that they cannot function independently of light chains in these organisms. The remaining question then is the function of KLCs.

II.C: Analysis of KLC Function

The observations from the gene knockout experiments are consistent with earlier predictions that the role of KLCs is either to regulate kinesin and/or mediate binding to organelles (Hollenbeck, 1993). The original suggestions were based largely on the observations that KLCs localize to the carboxyl terminus of kinesin (Hirokawa et al.,

1989), which lies outside the N-terminal motor domain. This end of the tetramer is not required for the translocation of kinesin along microtubules (Kuznetsov et al., 1989; Yang et al., 1990), and was therefore widely proposed to play a role in either binding cargo, regulating motor function, or both.

The KLCs were therefore indirectly implicated in these functions. Critical analysis of the literature with respect to these functions is discussed below.

i) Subcellular Localisation and Antibody Studies Suggests KLCs Contribute to Vesicle Binding

A large body of evidence based on immunolocalisation, cellular fractionation, *in vitro* motility and other techniques has established that kinesin co-localizes with MBOs, and functions to transport these in a plus end directed fashion. The best evidence supports a role for cytoplasmic transport of mitochondria, the golgi to ER compartment, lysosomes, vimentin and axonal transport of various MBOs (reviewed in (Bloom and Endow, 1994) (Goldstein and Philp, 1999; the kinesin home page).

Initial support for a role of KLCs in mediating cargo binding came from EM studies showing the localization of KLCs to the C-terminus of kinesin (Hirokawa et al., 1989). These results, in conjunction with subsequent computer analysis of rKLC-1 sequence suggested a fan-tailed protein structure which could be well suited as a linker molecule(Cyr et al., 1991). These suggestions were supported by experiments demonstrating that KLC antibodies could displace kinesin from vesicles (Yu et al., 1992). Similar experiments showed that antibodies targeted to the TPR domain of KLC inhibited fast axonal transport, suggesting that this domain might mediate binding of cargo (Stenoien and Brady, 1997).

In mouse KLC-1 knockouts, a subset of Kif5a did not co-purify with light chain in neuronal cells, in contrast to wild type. Furthermore, the localization of this pool of Kif5a was aberrant in these cells. The mislocalisation of Kif5a in this study suggests that KLCs are required for proper kinesin targeting. Therefore, the ability of KHC (from higher eukaryotes) to bind MBOs *in vitro*(Skoufias et al., 1994), may represent either only a subset of the kinesin-MBO binding activity *in vivo*, or a physiologically irrelevant function. Further evidence comes from subcellular localization studies of specific KLC splice isoforms in cultured hamster cells, as examined by both immunofluorescence and biochemical fractionation/western analysis. In these studies, antibodies which detected multiple KLC isoforms localized kinesin to multiple membrane bound vesicles and the diffuse cytosolic fraction. However, antibodies specific for haKLC-1 b/c or haKLC-1d/e isoforms showed that these localized primarily to mitochondria (Khodjakov et al., 1998) and golgi (Gyoeva et al., 2000) respectively, indicating that these splice isoforms may be specialized for particular cargoes within the same cell type. Taken in conjunction, these results support the view that separate KLC isoforms function to properly target kinesin to specific cellular cargoes

Interestingly, there are some conflicting reports on the role of kinesin with regards to mitochondrial and Golgi to ER motility, with some reports supporting a role for kinesin and others contradicting these claims. The basis of these contradictions is currently unknown, but it is interesting to speculate that the presence of multiple kinesin genes underlies them. For example, the expression of different KLC genes or splice isoforms amongst the different cell types analyzed could explain conflicting reports.

However, alternate explanations are also possible, such as differences in the expression of KRPs which function in the transport of these organelles amongst the cells examined.

ii) KLCs may regulate kinesin function

There is also evidence suggesting that KLCs regulate the mechanochemical function of kinesin. For example, an N-terminal 45kDa fragment of the kinesin tetramer and the KHC dimer alone both exhibit elevated MT stimulated ATPase activity (Hackney et al., 1991; Kuznetsov et al., 1989). As both of these molecules lack light chains, a possible explanation is that KLC can negatively regulate MT based translocation. A mechanism is suggested by observations that the kinesin dimer has a flexible hinge in the rod-like central domain (Amos, 1987). This hinge could explain the ability of kinesin to undergo salt-concentration dependent conformational changes: at physiological pH, kinesin is in a folded conformation, while at high salt concentration kinesin is in an extended conformation (Hackney et al., 1992; Hisanaga et al., 1989). Collectively, these results suggest that the C-terminus of kinesin can fold back via the hinge region and allows the light chains to impinge upon or regulate the activity of the N-terminal motor. This hypothesis is supported by co-transfection experiments, which showed that KHC transfected on its own localized to MTs. Co-transfected KLC co-localized with KHC and induced a shift from MTs to a more cytosolic localization. The authors present a hypothesis whereby binding of cargo and/or phosphorylation of KLC (explained below) induces conformational shifts in kinesin, which induce increases in MT affinity. The model is attractive because it integrates the evidence with the concept of regulation, which would allow targeting and efficient use of motor molecules (Verhey et al., 1998).

An alternate explanation is offered by a series of recent papers, which have identified a self-inhibitory property of KHC, independent of the light chain. The first study demonstrates that KHC is capable of assuming the folded and extended conformations in the absence of light chain. Deletion analysis implicates the C-terminal tail region as self-inhibitory: it interacts with the motor/stalk region to form the folded conformation, which has a reduced ATPase activity (Stock et al., 1999). A subsequent study demonstrates that hinge or tail deleted KHC is activated (as assayed by ATPase and motility assays) relative to wild type, and both mutants can be inhibited by a peptide consisting of the tail domain of KHC. KHC is also activated by binding artificial cargo (beads) (Coy et al., 1999). A final study shows that individual full length KHC molecules move more sporadically and less frequently on microtubules than KHC mutants which delete either the hinge or tail regions (Friedman and Vale, 1999). Collectively, these studies demonstrate convincingly that the hinge region of kinesin allows the molecule to fold back on itself, and allow a short C-terminal sequence to bind and inhibit the motor domain. Furthermore, they suggest an effective mechanism of regulation: free kinesin is folded and inhibited, cargo bound kinesin is a proficient ATPase. Additional evidence comes from KLC-1 knockout mice: KHC dimers lacking associated light chains (not present in wild-type mice) isolated from these mice had normal ATPase activity *in vitro*.

What then is the role of the light chains, if any, in regulating KHC activity? It may be noteworthy that the studies cited above were conducted *in vitro*, and may not accurately reflect kinesin dynamics *in vivo*. For example, one problem with the studies is that although folded KHC dimers have reduced ATPase and motility, they were still

capable of binding efficiently to MTs (Coy et al., 1999; Stock et al., 1999). This is in contrast to observations that the majority of kinesin is soluble within the cell, a lesser fraction associates with MBOs, and little or none localizes to the MT fraction (Hollenbeck, 1989b; Verhey et al., 1998). In fact, biochemical fractionation experiments indicate that stabilizing kinesin on microtubules requires the non-hydrolysable ATP analogue AMP-PNP, whereas in the presence of ATP kinesin preferentially localizes to the soluble fraction (rather than the MT pellet) (Vale et al., 1985a). Thus, a mechanism must exist which prevents kinesin from associating with microtubules. These observations have led to suggestions that self-inhibition of KHC may not suffice for accurate regulation and/or targeting, and extra regulatory factors such as KLCs could be required to ensure cytosolic localization of free (non-cargo bound) kinesin (Cross and Scholey, 1999). Thus, KHC self-inhibition does not rule out a regulatory function for KLC. However, further experiments are required to determine the exact nature of this regulation. For example, it would be interesting to determine whether the aberrant pool of kif5a in the KLC-1 knockout mice (described above) differed in its MT affinity *in vivo*.

iii) KLCs may be targets for Regulatory Signaling Cascades

One possibility, as suggested above, is that KLC may be the target for phosphorylation signaling cascades which regulate the binding of MBOs to kinesin, its targeting within the cell, and/or its mechanochemical activity. Golgi-ER traffic and axonal transport, for example, are both known to be mediated at least in part by kinesin (refer to reviews cited in introduction), and certainly these processes are subject to extrinsic regulation, (reviewed in Sheetz et al., 1989; Takenaka et al., 1998; Thyberg and

Moskalewski, 1999). Furthermore, as noted above, the majority of kinesin is known to reside in the soluble cytoplasmic pool of cells, rather than the MT pellet or the MBO fraction (Hollenbeck, 1989a). These same investigations established that kinesin is a phospoprotein *in vivo*. This led to suggestions that phosphorylation of kinesin could regulate its partitioning between these fractions and hence its function (Hollenbeck, 1993). An analysis of the relevant studies supports this view.

Studies in chicken neuronal cell culture established that both KLC and KHC exist as phosphoproteins in vivo. Of note, phosphorylation was not found on the motor domain of KHC, suggesting any regulation of the motor is indirect (Hollenbeck, 1993). Furthermore, this phosphorylation was found to be a dynamic process, and differs between soluble and membrane bound pools of kinesin (Lee and Hollenbeck, 1995). Additional studies showed that KLC is more highly phosphorylated than KHC, and this can be achieved by various kinases *in vitro* - most notably PKA and, to a lesser extent, PKC. Synthetic compounds which activate these kinases also induce KLC phosphorylation *in vivo* (Matthies et al., 1993). Finally, recall the presence of (putative) conserved PKC and PKA sites on KLC genes (Beushausen et al., 1993). Together, these results suggest that PKA and PKC may effect kinesin regulation by phosphorylation of KLC and/or KHC. However, other studies have ruled out PKA and PKC as the potential KLC kinases, at least in chicken neuronal cells (Hollenbeck, 1993). Thus, controversy exists as to which kinases are responsible for KLC phosphorylation. Interestingly, KLC has been shown to purify with both endogenous kinases, phosphatases (Lindesmith et al., 1997b; Matthies et al., 1993) and Calmodulin (Matthies et al., 1993). The identity of the

kinase(s) and phosphatase remains unknown. Regardless of the identity of the regulatory proteins, it seems plausible that KLC is a target for signaling cascades.

The implications of kinesin modification have been analyzed both *in vivo* and *in vitro*, also with somewhat conflicting results. In vitro phosphorylation of KHC and KLC by PKA was found to correlate with decreased binding of KHC to synaptic vesicles. (Sato-Yoshitake et al., 1992). In a crayfish neuronal model, activation of the PKA pathway correlated with increased KHC phosphorylation and the inhibition of anterograde transport of small MBOs (Okada et al., 1995). Although KLC phosphorylation by PKA was noted *in vitro*, the authors did not pursue this observation. Rather, KLC binding to vesicles was implied/assumed, and the relative contributions of each peptide thus remain unknown. However, these results do imply that phoshporylation of KHC is a negative regulator of MBO binding. In contrast, other studies showed that the membrane bound fraction of KHC was more highly phosphorylated than the soluble fraction, and that both KHC and KLC phosphorylation was increased in correlation with increased anterograde vesicle transport (Lee and Hollenbeck, 1995). Thus kinesin phosphorylation has been shown to correlate with both increased and decreased binding to MBOs. Perhaps these conflicting results are a consequence of the study of different vesicle fractions, different model systems, different kinases, different phosphorylation sites, or any combination thereof.

Additional studies have shown that the phosphatase inhibitor okadaic acid induces increased KLC phosphorylation, which correlates with increased motility of lymphocyte granules on microtubules *in vitro*. The increased motility was shown to be the consequence of increased affinity of the kinesin-granule complex for microtubule. The

authors conclude that KLC phosphorylation is a positive regulator of kinesin activity (Lindesmith et al., 1997b; McIlvain, Jr. et al., 1994). Thus, the phosphorylation of KLC has been found to correlate with both regulation of vesicle binding to kinesin, and with affinity of the kinesin-cargo complex with microtubules. Since these functions are not mutually exclusive, it remains a possibility that both are regulated by KLC. However, it will be necessary to sort out the individual contributions of the potential confounding variables noted above.

iv) Fungal KHC and the KRP family

Recall that fungi express KHC without associated light chains (Steinberg, 1997). This indicates that at least in these organisms, KHC is functional as a dimer - in terms of both regulation and cargo binding. Observations of KHC from higher eukaryotes capable of binding MBOs *in vitro* (Skoufias et al., 1994) and self-inhibition *in vitro* (Coy et al., 1999; Friedman and Vale, 1999; Stock et al., 1999)support the concept of a functional molecule in the absence of light chains. However, within the cellular context of higher eukaryotes, gene-targeting experiments clearly indicate kinesin is not functional without light chains. Therefore, these *in vitro* properties may reflect artifacts or minor KHC capacities, which are not sufficient for proper function in the more specialized cells of higher eukaryotes.

Additional hints regarding the role of KLCs come from study of the KRPs. Although well over 30 KRPs have now been identified in mammals, only KHC appears to associate with light chains (reviewed in (Bloom and Endow, 1994; Hirokawa, 1998). The accepted view is that the tail region of the KRPs mediates cargo binding, regulation and targeting of the motors. Thus, these mechanisms obviously exist and are functional

in the absence of light chains, although such mechanisms remain poorly understood. There are, however, possible analogies to KLCs as suggested by the co-purification of KAPs (Kinesin Associated Proteins) with some KRPs (reviewed in Goldstein and Philp, 1999). A good example is the KAP3 protein, which is associated with the KIF3A/B motor and - based on differences in its composition between brain and testis, is speculated to introduce functional diversity to the KIF3A/B motor (Yamazaki et al., 1995).

v) Summary of possible KLC functions

In summary, KLCs appear to be required for proper kinesin function in organisms which encode these genes. The high sequence conservation of KLCs amongst organisms implies specific, evolutionarily conserved functions. Their widespread expression and the evolution of multiple genes underline their importance to kinesin function. However, the role of KLCs remains controversial. There is good evidence that specific KLCs are involved in mediating interaction with specific cargoes. Furthermore, KLCs may be regulators of kinesin molecule function. A credible model is proposed by Verhey et al,1998, and integrates regulation and cargo binding functions. However, the available data does not conclusively implicate KLCs in these functions, and does suggest KLC independent mechanisms exist. Furthermore, the fact that the KRPs require similar regulation and targeting, but lack associated light chains, supports the idea that such KLC independent mechanisms exist. A plausible hypothesis is that of functional diversity: KLCs expand upon existing regulatory and targeting mechanisms to generate increased plasticity of kinesin function, regulation, and targeting.

III. UNUSUAL KINESINS: KINESINS WITH A NON-MOTOR FUNCTION

The paradigm of kinesins as microtubule motors is deeply entrenched, however the traditional view has been challenged recently by multiple observations. More recent reports have characterized KRPs which may not have a function in MT based motility, and consequently there have been admissions from investigators in the kinesin field that they often don't publish the failure to detect motor activity for kinesins (Goldstein and Philp, 1999). Thus, there is a slowly emerging view that other functions are also possible. An analysis of the literature also reveals multiple examples, that can be classified into distinct functions as follows.

III.a: Tethering of signaling cascades to the MT lattice

An emerging paradigm in the signal transduction field is that subcellular localization is a key predicator of function. There are several examples which suggest that KRPs may function to properly localize such molecules to MTs. In back to back papers in Cell, a group describes the characterization of *COS2*, a novel KRP. The gene had previously been implicated as a component of the Hedgehog (HH) developmental signaling pathway by genetic work. *COS2* was found to bind MTs independent of nucleotides, and exist in a complex with two other components of the pathway, the protein kinase *fused*, and the transcription factor *Ci*. The complex localized to MTs. The group proposes a model where HH signal leads to phosphorylation of the complex, perhaps by *fused*, and the subsequent release of COS2 from MTs. This would allow *Ci* translocation to the nucleus, thus effecting transcription of HH target genes (Robbins et al., 1997; Sisson et al., 1997).

A second example is that of the KRP *pavarotti* and the POLO kinase in *Drosophila* (MKLP1 and Plk respectively in mammals). The localization of the kinesin has been shown to coincide with that of the kinase on various components of the spindle including the centrosome, kinetochore and the spindle midbody. Furthermore, the proteins have been shown to interact (Lee et al., 1995; Adams et al., 1998). Based on observations that pavarotti mislocalizes in POLO mutants, and POLO mislocalizes in *pavarotti* mutants, it has been proposed that the proteins function to ensure their mutual localization. In the case of POLO, it is hypothesized that pavarotti ensures proper localization of the kinase to the spindle to allow phosphorylation of relevant targets (Adams et al., 1998; Carmena et al., 1998).

A further observation is that of colocalisation of MLK2 and MLK3 with members of the KIF3 superfamily of motor proteins. The interaction is potentially mediated via KAP3A, the putative targeting component of KIF3 motor complexes and KLC analogue of this KRP family member. The interaction was interpreted as a potential mechanism for activating/targeting KRPs in stress response (Nagata et al., 1998). The observation that -at least *in vitro*- KRPs can bind MTs but not display motor activity (Hanlon et al., 1997), suggests that such attachments need not involve motility, but could represent simple tethering. Thus, there is evidence that KRPs may function to properly localize kinases and other components of signaling cascades to MTs.

III.b: Control of Cytoskeletal dynamics:

The KRP family (but not KHC) has been extensively implicated in various aspects of MT assembly, dynamics, spindle morphology, etc. Various conformations of

the KRPs allow for functions such as zippering, sliding, cross-linking, bundling, etc. These functions largely engage the motor capacity of the KRPs (reviewed in (Joshi, 1998; Goldstein and Philp, 1999). However, there are recent reports of members of the Kin I family (XKCM1 and XKIF2), which categorically rule out a motor function for these KRPs. Instead, the authors show that the proteins bind to MT ends and - putatively by inducing conformational changes in the MT ends - catalyze release of tubulin dimers, leading to catastrophic disassembly of the MT. ATP hydrolysis by these KRPs does not have a motor function, but rather allows their dissociation from the tubulin dimer. This is paralleled by the observation that native kinesin can couple the movement of beads to the ends of depolymerizing MTs *in vitro*, and can affect the rate of MT depolymerisation (Lombillo et al., 1995). Thus kinesins may function to regulate the stability of MT ends, and may exploit this to generate non-motor related movements.

The above studies illustrate the pitfalls of interpreting sequence conservation and ATP hydrolysis as kinesin motor function. An illustration of this is the cloning of KifC2 by two groups. Both report MT binding activity, but cannot detect MT motor activity *in vitro*. The groups ascribe this to an *in vitro* artifact and assign KIFC2 a role in vesicle transport - based largely on localization experiments *in vivo* (Hanlon et al., 1997; Saito et al., 1997).

Kinesin has been implicated in the elaboration of the vimentin Intermediate Filament (IF) network. Studies have shown the movement of vimentin filament precursors along vimentin tracks, which could be inhibited by KHC antibodies (Prahlad et al., 1998). Consistent with this, the vimentin IF has been shown to parallel MT distribution in some cell types, and KLC has been proposed to mediate vimentin

molecule binding for transport (Gyoeva and Gelfand, 1991; Liao and Gundersen, 1998). However, KHC antibodies and modified tubulin, both of which inhibit vimentin transport, have also been shown to induce collapse of the vimentin IF to the perinuclear area (Gyoeva and Gelfand, 1991; Kreitzer et al., 1999). This is not entirely consistent with function consisting exclusively of vimentin IF precursor transport. Although alternative explanations are possible, these results suggests additional roles for kinesin, such as perhaps cross-linking and stabilization of the IF network.

III.c: Redundancy/overlap with actin/myosin function:

A current area of investigation is the overlap and relative contributions of the actin/myosin system and the kinesin-dynein/MT systems in mediating cellular traffic (reviewed in (Allan, 1995)). It has become apparent that there may be some overlap between these two transport systems, and thus it is not surprising that recently several KRPs have been cloned which embody this concept.

The first example is that of smy1p, a KRP (based on its sequence) which was originally cloned as a suppressor of the lethal myo2p mutation in yeast (Lillie and Brown, 1992). This myosin family member has been implicated in the growth of budding yeast cell daughters, putatively by transport of vesicles or by anchoring the actin cytoskeleton. Surprisingly, subsequent studies have shown both the colocalisation and physical interaction between myo2p and smy1p (Beningo et al., 2000; Lillie and Brown, 1994). Furthermore, studies demonstrated that smy1p does not appear to have an MT dependent function: perturbations of MTs or mutation of the putative MT binding domain in smy1p have no effect on its ability to rescue myo2p mutants (Lillie and Brown, 1998). The

authors propose that the two motors interact to form a functional complex, and-although the exact function remains unclear-it is MT independent (Beningo et al., 2000). Another link of sorts between the two cytoskeletal systems is presented by an unusual KRP cloned from *Arabidopsis*. Although this KRP is conventional in the sense that it appears to have a motor function in the mitotic spindle (Kao et al., 2000), it is unusual in that it encodes a myosin tail homology-4 region, and (also analogous to myosin) a calmodulin- binding region (Day et al., 2000). The presence of myosin motifs in KRPs and the overlap of KRP function with myosin function, from highly divergent organisms, underline the fact that these two cytoskeletal systems are somewhat redundant in function. It is quite possible that future work will reveal the presence of other motors with functions, which do not neatly partition to one of these two cytoskeletal systems.

III.d: Summary and Implications for KLC function

In summary, it is evident that kinesin and KRPs have cellular functions which do not involve the transport of cellular cargo on microtubules and/or a motor function. An important question raised by these observations is the construction of results uninterpretable or inconsistent with current paradigms by individual investigators: negative results are often not reported. The emergence of a body of evidence which offers alternate explanations for KRP function may hasten a broader and more accurate understanding of the potentially diverse functions fulfilled by this family of proteins. Put in the context of the concept elaborated above -that of functional diversity of KLC function - these findings raise interesting possibilities. As explained in the following sections, paradigms of kinesin function are derived largely from initial findings in

neuronal models. Interestingly, none of the unusual examples cited above involve neuronal systems. Thus, research in other tissues could conceivable uncover novel KLC functions.

IV. THE TESTIS AS A MODEL SYSTEM

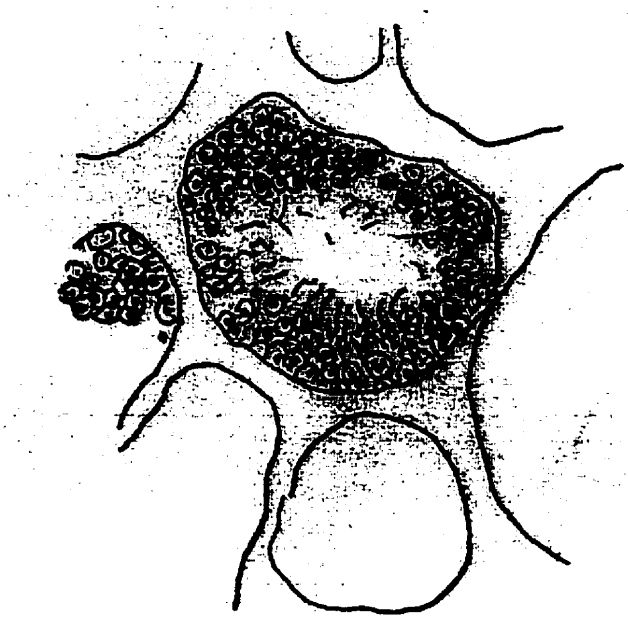
IV.a: Spermatogenesis and Spermatozoon Structure

Spermatogenesis is defined as the process during which a terminally differentiated sperm cell (spermatozoon) is formed from a stem cell (spermatogonium). This process begins at puberty and occurs continuously in the epithelium of seminiferous tubules (ST) in the testis. A cross section of the ST (see Fig.1) reveals that the spermatogonia reside at the outer edge of the ST. These embark on a differentiation program to produce spermatozoons. The process is highly synchronized, cells at different stages are spatially separated and morphologically distinguishable. Spermatozoons are released into the lumen, for passage to the epididymis for further maturation. In addition to differentiating sperm cells, Sertoli cells are also present in the ST, which function largely in regulation and support of sperm cells during their differentiation program. Interstitial cells and

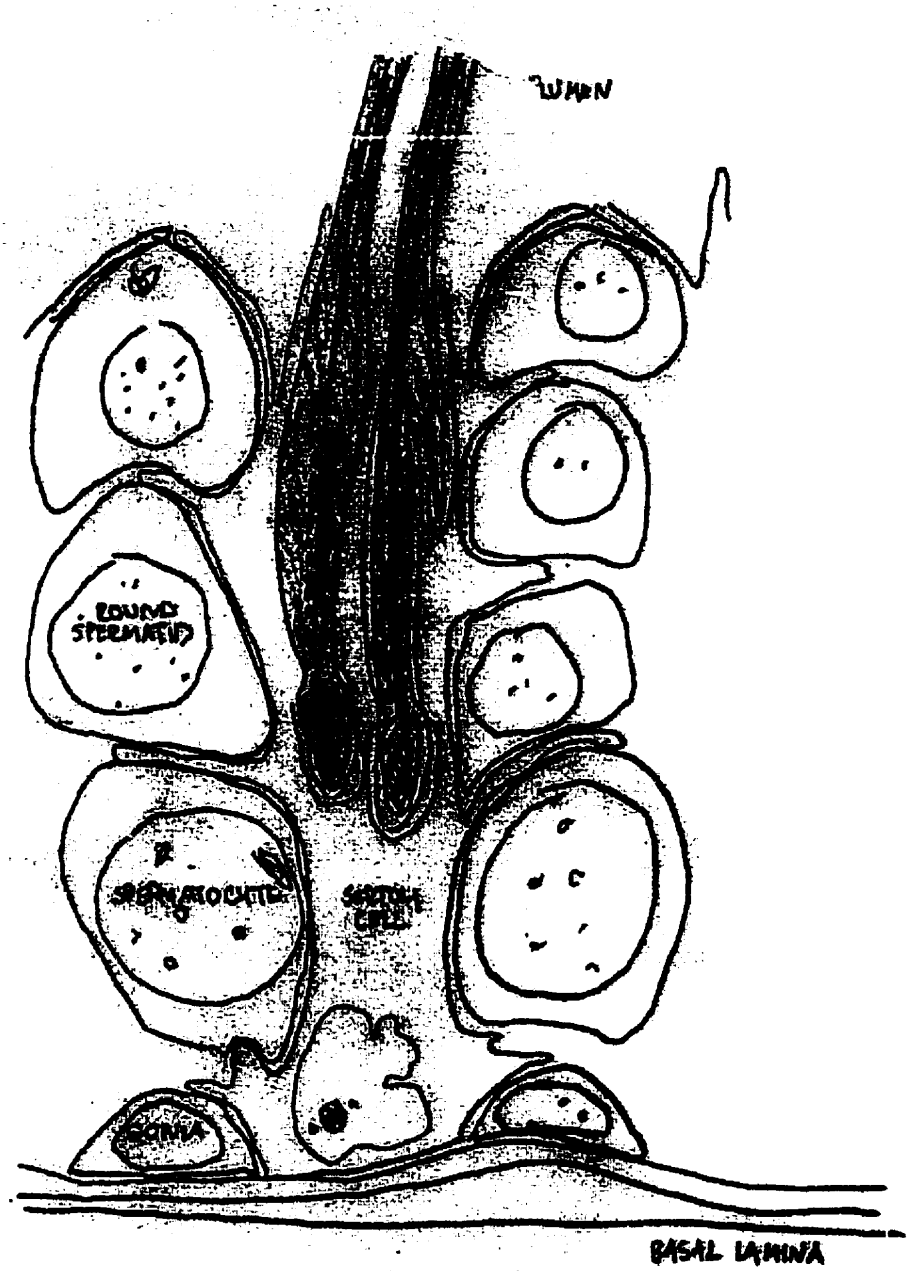
Figure 1

A) A schematic representation of a cross section of the human testis. From (Browder et al., 1991). B) A portion of the seminiferous tubule is shown, depicting the organization of cells. Note the spatial separation and relative positions of spermatogonia (gonia), spermatocytes, spermatids and elongate spermatids. Sertoli cells span the ST from the basal lamina to the lumen. Figure from (Russel et al., 1990).

1A



1B



blood vessels are also visible in testis cross-sections (Dadoune, 1994; Baltimore, 1990).

Undifferentiated spermatogonia undergo either mitotic divisions to renew their own population, or enter differentiation. Initial differentiation includes DNA duplication (to 4N chromosome number) to generate primary spermatocytes. These undergo the first meiotic division, giving rise to secondary spermatocytes. The second meiotic division generates spermatids (1N). Spermatids embark upon morphological differentiation into spermatozoons in a process known as spermiogenesis. Spermiogenesis has been extensively characterized, and in the rat can be divided into 16 stages based on characteristic morphological features (Fig. 2). Major adaptations of the mature sperm cell include a lack of ribosomes, endoplasmic reticulum, Golgi Compartment and minimal cytoplasm. The DNA in the nucleus is highly condensed by protamines, not histones, does not support transcription, and is surrounded by a thin, dense cytoplasmic layer known as the perinuclear theca. The acrosome is tightly attached to this, and contains hydrolytic enzymes for zona pellucida (oocyte coat) penetration. Finally, mature sperm have a flagellum. Thus, the sperm cell is highly specialized for the singular task of delivering its DNA content to the female egg(Dadoune, 1994; Baltimore, 1990).

IV.b: The Sperm Cytoskeleton

Aside from the axoneme, the manchette is the other prominent MT structure in elongating spermatids. The manchette is a transient MT structure which surrounds the nucleus and may mediate the condensation of the nucleus to its characteristic shape, and

2

Figure 2: The staging of germ cell development in the mouse. The Individual stages are explained in more detail in Russel et al, 1990, from which this figure is adapted. Shown here is the designations given to mouse Sperm cells with the shown morphologies, at various stages of development.

is disassembled later in spermiogenesis (Russell et al., 1991; Soley, 1997). Additional functions proposed for the manchette include movement of cytoplasmic elements such as vesicles (Rattner and Brinkley, 1972). However, the manchette remains poorly characterized.

The flagellum consists of a central axoneme, the outer dense fibers, the fibrous sheath and numerous mitochondria which power flagellar beating (Fig. 3). The central axoneme is a standard ciliary/flagellar 9 + 2 axoneme, but with testis specific alpha/beta tubulin subunits (Distel et al., 1984). A large number of additional proteins are thought to reside in the axoneme (Gibbons, 1981). The tail can be divided lengthwise into the connecting piece, the midpiece, the principal piece and the tail. The connecting piece is short electron dense, largely cytoskeletal structure that adjoins the head and the flagellum. In the midpiece of the flagellum, the axoneme is surrounded by 9 outer dense fibers (ODFs), each associated with a MT doublet. Elongated, helically wrapped mitochondria surround the midpiece/ ODFs. In the principle piece, ODFs 3 and 8 (which represents a plane through the central axoneme MT doublet) are replaced by the Fibrous Sheath (FS), which are longitudinal columns bridged by transverse ribs. The ODFs and FS taper principle piece, and in the tail of the flagellum only the axoneme is present(Fawcett, 1975).

The ODFs and FS are cytoskeletal structures unique to spermatids, and their constituent proteins are thought to be spermatid specific in expression (Oko and Clermont, 1989). The two differ somewhat in composition, but share immunological and biochemical protein similarities with each other (Oko, 1988), and to a lesser extent with

the perinuclear theca and the connecting piece. The ODF and FS cytoskeleton is an SDS insoluble structure, likely due to high disulfide bond content. The ODF is composed of 6 major polypeptides, with additional minor constituents(Oko, 1988) (Vera et al., 1984). The FS is composed of 18 polypeptides, 3 of which are in large abundance, with others in intermediate or minor abundance (Oko, 1988). The function of the ODF and FS is unclear, but may involve mechanical aid to flagellar beating, protection against shear forces in ejaculation, and/or as an attachment site for the helically wrapped mitochondria. A number of the constituent proteins of ODFs and FS have been cloned and characterized. Relevant to this study are ODF-1, ODF-2, and SPAG 4.

IV.c: Spermatid Cytoskeletal proteins ODF1, ODF2 and SPAG4

The first ODF protein to be cloned was ODF-1 (van der Hoorn et al., 1990; Burfeind and Hoyer-Fender, 1991; Morales et al., 1994). Characterization of the protein has demonstrated that it has an N-terminal leuzine zipper which mediates weak self association in vivo, and 16 C-terminal CGP repeats highly reminiscent of the *Drosophila* MST family. It is spermatid specific and localizes exclusively to the sperm tail (van der Hoorn et al., 1990), in the ODF, and more specifically to the medulla of the ODF (inner side facing the MT doublet) (Schalles et al., 1998). Using ODF-1 as bait in yeast two hybrid screens, two ODF-1 interacting proteins have been cloned, ODF2 and SPAG4. ODF2 dimerizes with ODF1 via its N-terminal leucine zipper, is expressed exclusively in spermatids and therein only in sperm tails (Shao et al., 1997). It is synthesized in

3

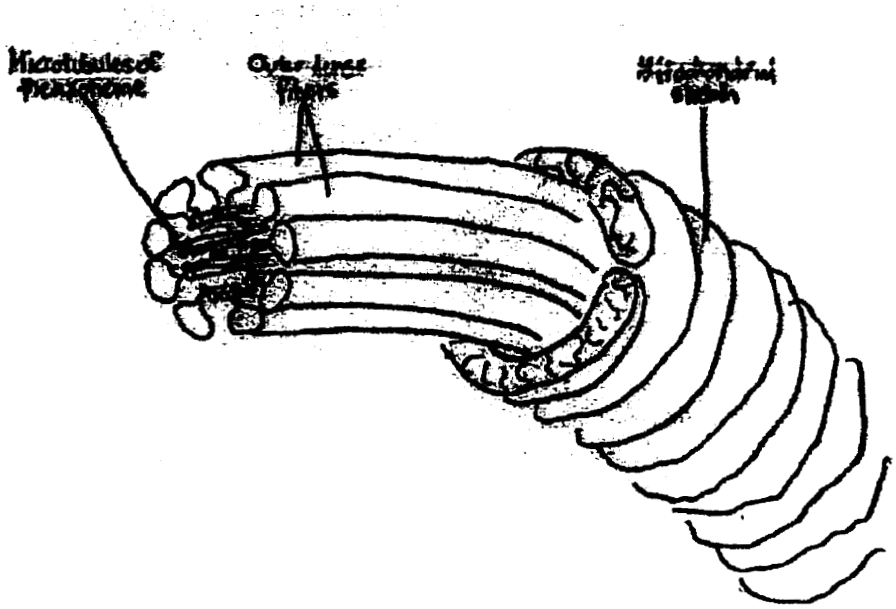


Figure 3: A schematic diagram of a cross-section through the mid-piece of the sperm flagellum, showing the mitochondrial sheath, the outer dense fibres and the axoneme.

elongating spermatids and assembled into the developing ODFs in a proximal to distal fashion, and into various cytoskeletal structures in the connecting piece. It is found in both the cortex and medulla of the ODF, but not in the FS (Schalles et al., 1998). SPAG4 also interacts with ODF1 (but not ODF2) via its leuzine zipper and is spermatid specific. It has a SAD1 homology domain (SAD1 is a MT binding protein in yeast speculated to aid motor protein binding to MTs), and interestingly localizes to MT structures - both the manchette and the axoneme. It is not clear if this is mediated by the SAD1 homology region. It is speculated that SPAG4 acts as a link between ODFs (specifically ODF1) and the axoneme and may aid in ODF1 localization to the medulla of the ODF. The yeast two hybrid system was used in the study of interacting domains, and is especially useful due to the insoluble nature of these proteins, which poses unique challenges *in vitro*. In this context, it is important to note both that leucine zippers are predominant in ODF protein interactions, and further that these appear to be very specific: for example, the leucine zipper of ODF2 interacts with that of ODF1, but not that of SPAG4. It is expected that cloning and characterization of further ODF and FS proteins will aid in developing an understanding of the function of these structures.

IV.D: Kinesins in the testis

Despite the fact that the testis should express a large number of MT motors for its various MT structures, the characterization of kinesin and KRPs in this tissue remains minimal. Early evidence for the importance of MT transport in the testis comes from the observation that testis is an excellent source of cytoplasmic dynein (Collins and Vallee,

1989; Neely and Boekelheide, 1988). KRPs have also been reportedly expressed at high levels in the testis, such as the heterotrimeric kinesin II family member Kif3A/B - KAR3 (Yamazaki et al., 1995). The prevalence of KRPs in meiotic processes predicts a large diversity of KRPs in the testis. In a search for such kinesins, a group has cloned six novel KRPs, 5 of them predominantly testicular in expression, most of which colocalize to meiotically active regions of the rat SE (Sperry and Zhao, 1996). Two of these have been investigated in more detail. KRP2 has been identified as a Kif2 family member and is expressed predominantly in meiotically active cells (Sperry and Zhao, 1996). The other, KifC5a and/or antigenically related peptides have been localized to the spindle of meiotic cells in the testis (Navolanic and Sperry, 2000). In *Drosophila*, two other KRPs have been assigned spindle functions: KLP3A (Williams et al., 1995) and *pavarotti* (Adams et al., 1998). Considering that meiotic and mitotic spindles movements are complex and require the co-ordination of many motors, much work remains to be done to characterize these in the testis. However, these may not be very different from those involved in other cell types: cell division movements appear to be highly conserved.

On the other hand, the manchette, the sperm flagella and the Sertoli MT network offer more unique challenges for motor characterization. Immunolocalisation and biochemical fractionation experiments have localized Kif5b heavy chains to the trans Golgi network in Sertoli Cells (Johnson et al., 1996a; Hall et al., 1995), and the manchette in spermatids (Hall et al., 1992). Immunolocalisation to the Golgi is consistent with a role for KHC in Golgi trafficking (reviewed in(Bloom and Endow, 1994; Andrews et al., 1993; Goldstein and Philp, 1999). Localisation to the manchette raises the possibility that this MT network is involved in vesicular trafficking: in line with this,

vesicles have been visualized in association with manchette microtubules (Rattner and Brinkley, 1972). However, the function of the manchette is not known, and thus Kif5b may fulfill a different function here. KifC5a has also been localized to the manchette (Navolanic and Sperry, 2000).

Finally, there are also reports of kinesins in the sperm tail. In addition to KifC5a, kinesin II family members have localized to the sperm tail in the rat (Miller et al., 1999) and sea urchin/sand dollar (Henson et al., 1997). The report in echinoderms is interesting as it reports kinesins in both the flagellum and the midpiece. In the midpiece, the staining is similar to centrosomal staining. The staining was not detergent extractable, indicating that the kinesin II was not membrane associated, and further noted a punctate or discontinuous staining pattern with their antibody. Based on the similarity of these results to those obtained in Chlamydomonous with FLA10 (subsequently also identified as kinesin II), the authors speculate similar function in the two organisms for the protein homologues. The work in *Chlamydomonas* is especially interesting: pioneering work in this model organism has led to the realization that flagellar microtubules are themselves involved in the transport of components required for assembly and maintenance of these structures. Furthermore, this process requires MT motors and is highly regulated (reviewed in (Joshi, 1998). For example, Kinesin II has been implicated in the transport of intraflagellar transport particles (known as rafts) in *Chlamydomonas* (Cole et al., 1998). These rafts differ from MBOs, and may circulate as carriers of flagellar structural constituents such as inner dynein arms (Piperno and Mead, 1997). The paradigm may well be true for flagellar structures in higher eukaryotic organisms. Although there is no experimental evidence supporting or refuting this concept for kinesins in sperm flagella,

the presence of kinesin II family members in the sperm tail is highly suggestive (refer to the kinesin home page for more information on IFT).

IV.e: We propose the testis as a good candidate model system to study Kinesins

Germ cells provide an excellent system to model the basic cellular processes of differentiation and development. Spermatogenesis is highly temporally and spatially organized. Maturation from diploid stem cell to spermatozoon can be visualized in cross section of the mature tubule, and involves cells at various stages of mitosis, meiosis and morphological differentiation. The process is synchronized, and cell type/stage of differentiation can be classified by visualization. Cellular differentiation into mature spermatids includes the processes of meiosis, mitosis and dramatic morphological changes, including changes in the distribution, abundance and presence of various organelles and cytoskeletal structures. This can be exploited to analyze the expression of proteins in different stages of differentiation by coupling microscopy with immunological techniques. Furthermore, centrifugal techniques can be used to isolate cells at distinct developmental stages (Higgy et al., 1995a), thus allowing for biochemical analysis of defined cell types. Consequently, the testis is an easily accessible *in vivo* model system, and has been exploited extensively for the study of the molecular mechanisms underlying cell differentiation and development.

In conjunction with the presence of the multiple unique MT structures that exist in the testis (manchette, flagellum, meiotic and mitotic spindles, etc) these properties seem ideal for the study of kinesins. However, most work on kinesins has been done in neuronal systems. Neurons, due to their long processes, have specialized transport needs:

synthesis of proteins and other axonal/dendritic components such as synaptic vesicles occurs in the cell body, not the axon. It appears that the MTs and their associated motors bear the burden of transport of such axonal components. As a consequence, neuronal cells express high levels of motor proteins. Kinesin was originally purified from neuronal cells (Brady, 1985; Vale et al., 1985a) and initially its function (and that of many KRPs) was largely determined by immunolocalisation, inhibition and biochemical fractionation experiments in neuronal cells (reviewed in Brady, 1991). Research in other tissues has largely borne out the concept of kinesin as a cytoplasmic motor for organelle transport. However, these findings cannot be unequivocally generalized to all cell types. The testis offers various unique MT structures not represented elsewhere in the body. Intriguing parallels and unexpected differences have been identified between intraflagellar transport and axonal transport, and similar patterns may emerge for sperm flagella. The function of the manchette remains poorly characterized. The localization of kinesins to both of these structures merits further investigation of their function. Furthermore, the emerging concept of kinesins with unusual functions provides additional impetus for examination of novel tissues, especially those with unique cytoskeletal structures.

As described above, the cellular and subcellular expression patterns of KLCs have lend credence to claims regarding the role of KLCs in mediating cargo binding to kinesin, and further support the concept of functional diversity of KLCs. If the hypothesis of KLC gene specialization is correct, one would for example expect the abundance, expression patterns and subcellular localization of the individual genes to parallel these same properties in their cargo. Since the testis offers easy visualization and

isolation of differentiating cell types, it may be a good model system to study expression patterns of kinesins. The drawback to the use of the testis as a model is the absence of a tissue culture system. Thus, experiments requiring such a system have to be conducted either *in vitro*, in other cell types, or by gene knockout.

V. THE C-MOS ONCOGENE AND IT'S LOCALISATION TO MICROTUBULES

Mos was the first serine/threonine kinase oncogene to be discovered, initially as a viral oncogene from the Moloney Murine Sarcoma virus (Van Beveren et al., 1981), and subsequently cloned from various species including *Xenopus*, mice, humans and rats (Watson et al., 1982; van der Hoorn et al., 1982; Van Beveren et al., 1981; Sagata et al., 1988). As such, it has been extensively studied both in regards to its role as an oncogene and its endogenous function in the cell. c-Mos is expressed at very low levels in a variety of tissues, including placenta, brain, kidney and muscle, and at higher levels in germ cells - both oocytes and testis (Propst et al., 1987). The role of c-Mos in oocyte maturation is well defined. In *Xenopus* oocytes, c-Mos is a key regulator of the cell cycle, and is required for the activation of Maturation Promoting Factor (MPF), which stimulates the onset of oocyte maturation, subsequent re-activation of MPF to ensure proper passage to meiosis II, and finally as a component of Cytostatic Factor (CSF), which stalls oocytes at metaphase II until fertilization. In mouse oocytes, c-Mos is also a component of CSF and may aid in the transition from meiosis I to II, however it is not required for activation of MPF. c-Mos exerts its influence in oocytes by phosphorylating and activating MAPKK and thus the MAPK signaling cascade. (see Singh and Arlinghaus, 1997; Gebauer and Richter, 1997 for reviews). The role of c-Mos in male germ cells and in other tissues is

not clear, as male mos-/- mice are fertile and furthermore have no detectable phenotype (Hashimoto et al., 1994; Colledge et al., 1994)). However, overexpression of c-Mos in testis results in elevated levels of germ cells, suggesting that it may play a role in cell-cell communication (Higgy et al., 1995b). In myoblast cells, c-Mos may contribute to myogenic differentiation (Lenormand et al., 1997). Thus, c-Mos may play different roles in different tissues.

In addition to its role as CSF, Mos has been extensively investigated for its cell transforming capacities. Ectopic expression of Mos in somatic cells results in cellular transformation (Blair et al., 1980), and it appears that Mos may function by various mechanisms to achieve cellular transformation: these include deregulation of G1/S (Sagata, 1997) and G2/M (Laird and Shalloway, 1997) checkpoints, or by imposing a meiosis like phenotype upon somatic cells (Fukasawa and Vande Woude, 1995). Controversy persists with regards to which functions contribute to cellular transformation. Two consistent themes how are the ability of c-Mos to influence the cell cycle by various mechanisms, and the dependence of c-Mos induced cellular transformation on the presence of c-Fos.

To understand Mos function, it has been necessary to delineate partitioning of Mos and its targets within the cell: c-Mos has been ascribed differing sub-cellular locations -associated with different function- in the various tissues where it has been described. For example, in testis c-Mos is found in large insoluble complexes (Nagao, 1995a), in oocytes it is associated with tubulin (Wu et al., 1997), and in muscle cells it is found in a soluble cytoplasmic fraction (Leibovitch et al., 1993). In transformed somatic cells, c-Mos associates with microtubules (MTs) (Zhou et al., 1991a; Zhou et al., 1991b;

Bai et al., 1992; Rulong et al., 1995) as well as the nucleus (Fukasawa et al., 1995) and kinetochores (Wang et al., 1994). Mutations that decrease nuclear localization increase transformation capacity of c-Mos (Fukasawa et al., 1995). Thus, it has been speculated that localization of c-Mos to microtubules may be necessary for cellular transformation to occur. This hypothesis is consistent with the observation that MAPK can localize to MTs and the spindle, and its role as a G1/S phase regulator. In addition, the presence of c-Mos on microtubules may have functional implications on the mitotic spindle. c-Mos has been shown to phosphorylate tubulin (Zhou et al., 1991a; Zhou et al., 1991b), and mos-/- mouse oocytes develop abnormal spindle morphologies (Verlhac et al., 1996). Injection of Mos antibodies into oocytes has a similar effect on the spindle. Thus colocalisation of c-Mos with microtubules may be an important predicator of its ability to both transform cells and influence spindle morphology. (A good parallel for this situation is given by the POLO kinase, described in the previous section on unusual kinesins - kinase tethers).

VI. SUMMARY OF PREVIOUS RESEARCH ON KLC-3

The following is a summary of research on KLC-3 conducted by members of the van der Hoorn lab, in collaboration with the Oko lab (van der Hoorn, per coms). To gain a better understanding of the function of Mos in the testis, kinase dead c-Mos was used as bait in a yeast two hybrid screen of a rat testis cDNA library. Among the interacting proteins was a novel gene. Initial BLAST searches revealed >90% sequence homology to an open reading frame in the ERCC2 locus, which has been identified and sequenced in human, mice and hamster. The three genes encoded in the ERCC2 locus are gene A, a putative transcription factor, ERCC2 involved in DNA damage repair and a putative KLC

gene, based on ~50% sequence similarity to the sea urchin kinesin light chain (Lamerdin et al., 1996). The novel gene was called KLC-3 as it represented a potential novel member of the KLC family. Initial efforts focused on the relevance of the interaction between c-Mos and KLC-3. *In vitro* phosphorylation assays of KLC-3 by c-Mos were unsuccessful, suggesting instead that KLC-3 might act as a MT tether for c-Mos.

Subsequent characterization of KLC-3 focused on analysis of its expression pattern. Northern blots failed to detect KLC-3 in brain, kidney or liver, but did detect it in testis, and therein in spermatids but not spermatocytes. Monoclonal antibodies were raised to an MBP/KLC-3 fusion protein, and one of these (11g6b2) generated a signal specific to the tails of elongating spermatids and mature spermatozoa in immunofluorescence experiments. The signal was not detergent extractable, and furthermore, KLC-3 signal remained associated with sperm tail structures in the fertilized oocyte (mitochondria and other structures are quickly stripped from the sperm upon fertilization) This suggested that at least a subset of KLC-3 is not membrane associated in sperm tails. The same antibody was subsequently used in immunogold/EM experiments, which showed expression specific to elongating spermatids/spermatozoa, and association with mitochondria, the submitochondrial reticulum, the cortex of ODFs, and also MTs of the axoneme.

VII. Hypothesis/ Research Aims:

It is difficult to predict the function of this novel clone, KLC-3, when the precise function of the more widely studied KLCs remains unclear. Determining the function of a protein requires more research than can be conducted during the course of an MSc. . Upon my arrival in the lab, it was hypothesized that KLC-3 was a novel, testis specific, non microtubule based, Kinesin Light Chain with a novel role in the sperm tail. Therefore, the purpose of this study was a preliminary characterization of the sequence, expression pattern and potential interacting proteins of KLC-3. As described in subsequent sections, the investigations described herein have contributed to a better understanding of these aspects of KLC-3.

Research Question	Investigations conducted
Is KLC-3 a KLC?	-sequence analysis/genomic localisation -interaction with KHC *(in vitro)* -interaction with microtubules *(in vitro)*
What is the function of KLC-3 in the testis?	-determine expression pattern within the Seminiferous tubule (of KLCs and KHCs) -determine subcellular localization of KLC-3 and KHC isoforms within sperm cells -identify potential interacting proteins based on results of immunolocalisation

MATERIALS AND METHODS

PCR analysis of genomic DNA and genomic clones to determine linkage with ERCC-2

Genomic clones and mouse tailsnip DNA was obtained from Bhupinder Bhullar, University of Calgary. Rat genomic DNA was obtained from Heide Tarnasky. The primers were designed based on both the published sequence for ERCC2 (Lamerdin et al., 1996), and the sequence of KLC-3 cDNA (van der Hoorn, per coms). Primers were: CAACCTGACCGTGGATGAG -3', ERCC2-3' (exon 22) 5'GTGTCTC CTCTG ACTGCAG-3', KLC-3 -5' (exon 13) 5'-GCTCAGATCTTGTGTGCTG -3', KLC-3-3' (exon 12). 5'ATG CTG AAC G TG GAT GGT C-3'. ERCC2-5' (exon 21). PCR was performed using standard protocols and a Perkin Elmer 9600 cycler. The PCR reaction contained 1X Taq buffer (GIBCO BRL), 2.0 mM $MgCl_2$, 20 pmol of 5' and 3' primers, 1 % DMSO, 0.2 mM dNTPs, 1 ng DNA template and 1 U Tsg polymerase. Subsequently, 5'- 10 □l of the 25 □l PCR reaction was analyzed on 1.2% agarose gels. Marker DNA was phage lambda DNA digested with PstI. Digestions were performed by ethanol precipitation of PCR product, followed by resuspension water. Then, 2ul were added to water and the appropriate amount of the requisite 1X Bohringer-Mannheim restriction buffer to achieve 1X concentration. 1 unit of the requisite restriction enzyme was then added, and the reaction incubated at 37C for 1 hour. The reactions were then analyzed by agarose gel (1.7%) electrophoresis.

Sequence analysis

The KLC-3 cDNA was previously sequenced in our laboratory (van der Hoorn, per coms) (GenBank accession number for KLC-3 is AF166267). All sequence alignments, dendograms and pairwise sequence comparisons were generated using OMIGA software. The same program was used for the conceptual translation of KLC-3. Sequences for all other organisms were obtained from NCBI. These protein sequences were then used to generate the alingments in OMIGA using default parameters, except for the color coding of the alignment figures, which was set to highlight mutation frequency. The dendograms and pairwise sequence alignments are automatically generated with the alignment. For the HR, TPR and PEST region analysis, the relevant regions of KLC-3 were identified from regions of high homology in the sequence alignment. The same regions of mKLC-1, mKLC-2 and rKLC-1 were based on published sequence analysis (Rahman et al., 1998; Cyr et al., 1991). Predicted secondary structures were generated using the GOR II or Chou & Fassman algorithms present in the OMIGA menu. Protein motifs and phosphorylation sites were also found using OMIGA menu options.

Transcription, Translation and Immunoprecipitation with Kif5c

Kif5c was a gift of Dr. Schnapp, and was provided in the pBluescript vector with a region of the 5' UTR, cloned via Ecor1 sites. The first two features allow for *in vitro* translation. KLC-3 cDNA was previously cloned in the pCi vector in our lab (van der Hoorn, per coms). 2ug of total plasmid, either alone or split between the two plasmids at 1:1, 1:3 or 1:6 ratio of KLC-3 to KHC, or no plasmid control, was translated using the TNT system from PROMEGA. The manufacturers recommended conditions were used

(^{35}S-cysteine was used as a radioactive label for the proteins). All translation reactions were next immunoprecipitated using 5 ul of anti HA tag MAb in no salt lysis buffer, specific for the KLC-3 cloned into the pCi vector which has an HA tag (both antibody and vector gift of Dr. Young's lab). Immunoprecipitated proteins were analyzed on 10% SDS-PAGE mini-gels (Biorad system), which were then dried and analyzed by autoradiography. The SPAG4/KLC-3 interaction was analyzed in exactly the same manner. The HR deletion mutant was generated by Heide Tarnasky in our lab. The KLC-1 clone was a gift of Dr. Goldstein, and was subcloned into the same pCi-HA tag expression vector using Eco R1 and Sal 1 sites. c-Mos was cloned into the same vector by Bhupinder Bhullar. KLC-1, KLC-3, KLC-3 HR deletion, Mos and KHC were translated individually or co-translated with KHC using the TNT system (Promega) at a 1:1 ratio of plasmid DNA's. The immunoprecipitation, SDS-PAGE and autoradiography were conducted as above.

Microtubule binding assays

MTs were purified from Brain or testes as described by Vallee and Collins, (1986). Brain and testes samples were extracted in BRB80 buffer containing protease inhibitors (pepstatin, leupeptin, PMSF, DTT). The extract was centrifuged at 55,000rpm for 15 minutes at 4 °C. The supernatant was removed and stored on ice. The extracts were supplemented with 0.5 mM GTP and 15 U/ml hexokinase and 20 mM D-glucose to deplete ATP. After the extract was warmed to 30 °C, 5 □M taxol was added and mixed. After a 5 min incubation at 30 °C, 15 □M taxol was added (20 □M total). AMP-PNP (2 mM) was added to stabilize KHC on MTs. The extract was layered onto a sucrose

gradient and spun 40,000rpm for 20 minutes at 22 °C. The cushion was washed 2X with BRB80 before removing. The MT pellet was resuspended in 100 □l BRB80 and MTs depolymerized on ice for 20 min. GTP (0.5 mM) and AMP-PNP (2mM) were added to the tubulin fraction. The tubulin was warmed to 30C and polymerized and centrifuged as above. The pellet was subjected to another round of depolymerization and polymerization as above. The final pellet of polymerized MTs was resuspended in 10% glycerol/BRB80 and flash frozen in liquid nitrogen. To test if KLC-3 binding to MTs was sensitive to nucleotide concentration, KLC-3 DNA was *in vitro* transcribed and translated in the presence of ^{35}S-cysteine using the TNT reticulocyte transcription and translation system (Promega) as described above. The labeled proteins were incubated with polymerized MTs at 30 °C for 15 min in the presence of AMP-PNP (2 mM), GTP (0.5 mM) and taxol (20 □M). The MTs were pelleted at 30,000 rpm for 15 min at 22 °C. Supernatants were saved for gel analysis. Two subsequent pelleting reactions were performed with either AMP-PNP (2mM), or ATP at 2.5, 10 or 20mM. Aliquots of both supernatant and pellets were boiled in SDS sample buffer and analyzed by electrophoresis on 10 % SDS-PAGE gels, the gels dried and these exposed to film.

BLAST searches

Searches for potentially novel KLC genes were conducted using full length KLC-3 and KLC-1 cDNA and nucleotide sequences using advanced BLAST. The genomes for Drosophila, E. coli, yeast and C. elegans, mouse EST's and the partially completed human genome can be accessed from the same site and were also searched. Matches were subjectively determined using the two parameters given by the search algorithm,

namely the identity/length of the matching sequence, and the E value (expected number of chance hits with the same identity and length). Not all good matches are shown due to space constraints and the disorganized nature of EST and genome sequences.

Germ cell extracts and RNA isolation

Germ cell were isolated by Heide Tarnasky as follows: briefly, testes were isolated from two RT7/c-mos transgenic mice. Seminiferous tubules were treated with collagenase/trypsin to release Sertoli cells and germ cells, which were fractionated by centrifugal elutriation as described previously. Fractions were obtained of 90 % pure pachytene spermatocytes, round spermatids and late spermatids. Total RNA was isolated from these and from total testis, liver, brain, kidney, ovary, small intestine, heart, spleen and lung using the guanidinium-isothiocyanate method. RNA from each tissue was quantified by spectrophotometry

RT PCR

The reverse trancsriptase reaction was set up as follows (20 ul reaction): 5ug total RNA, 1ul random hexamers (500ug/ml) and sterile water to 11ul. Heat to 70C, chilled on ice, 4ul first strand buffer added (Gibco Brl), 2ul DTT (0.1M), 1ul dNTPs (10mM of each dATP, dCTP, dTTP,dGTP). and 1ul of Superscript RT (Gibco Brl). The cDNA was used as a template for the PCR as follows: All of the PCR reactions contained 1X Taq buffer (GIBCO BRL), 2.0 mM $MgCl_2$, 20 pmol of 5' and 3' primers, 1 % DMSO, 0.2 mM dNTPs, 1 ng DNA template and 1 U Tsg polymerase. 5'- 10 □l of the 25 □l PCR reaction was analyzed on a 1.2% agarose gel. All PCR programs were: 94C for 5 min, then 40X(94C for 60 seconds, anneal at X for 30 seconds, extend at 72C for 60 seconds), 72C for 10 minutes, 4C hold. Primers and annealing temperatures were as follows:

KLC-3 (primers 609 (tgg tga acc act cgc tg) and primer 834 (gtc gac g(c/g)t gag gat ctc ctt gta), anneal at 54C), ERCC-2 (primers 600 (gct cag atc ttg tgt gct g) and 601 (gtg tctc ctc tga ctg cag), anneal at 60C), actin primers (refer to (van der Hoorn, per coms)anneal at 58C), KLC-2 (primer 922 (acg atg gtg ctt cct cga) and primer 923 (a tct tca tcc agc ttt cgg), anneal at 55C), KLC-1 primer 657 (atg cat gac aac atg tcc ac) and primer 682 (cag cgg aca gca ctg gag) anneal at 58C), Kif5a 5' primer (agc tgg cgg tca act acg) and 3' (gc ctt ctt cac c tcc tcc) anneal at 56C), Kif 5b, primer 1156 (aac tgg ctg tta att atg) and 1157 (tc aac agc ttg ctt gact) anneal at 50C), Kif5c (primer 924 (agc tgg ctg tca att acg) and 925 (tc cag agc ttt ctt cac c)(anneal at 57).

Western analysis of KLC-3 and KHC expression

Elutriated germ cells were prepared by Heide Tarnasky as described above. These were lysed in lysis buffer containing 50 mM Hepes pH 7.0, 1% Nonidet-P40, 1 □g/ml Aprotinin, 100 □g/ml PMSF. Testis and brain tissues were flash frozen in liquid nitrogen and ground with a mortar and pestle. The tissue (still frozen) was then homogenized in a dounce homogenizer in lysis buffer. Samples were standardized by first quantifying for both total protein and total nucleic acid by spectophotometry. Subsequently, this was verified by comparing relative levels of background binding to secondary antibodies in control Western analysis. All Westerns were conducted as follows: samples were run on 10% SDS-PAGE gels (biorad system) in laemli buffers, and transferred to nitrocellulose membranes. These were blocked in low detergent buffer (1XTBS containing 0.1% NP-40 and Tween 20 and 5%BSA), incubated with primary antibody in the same buffer, washed three times, incubated with secondary antibody, washed three times and then developed using the ECL system buffers and exposed to film. All Westerns were done in

duplicate, both with and without a primary antibody. Antibodies and their concentrations were: KLC-3: polyclonal antiserum #19, made by Heide Tarnasky and pre-immune serum #19, used at 1:200. Secondary antibody was goat-antirabbit-HRP at 1:20,000 courtesy of the Steve Robbins lab. KHC antibodies H1 and H2: commercially available from Chemicon, and both were used at 1:300 dilution. Tubulin antibodies: commercially available from SIGMA, used at 1:300. Secondary antibody for KHC and tubulin was sheep anti mouse Ig POD from Bohringer Mannheim at 1:20,000.

Immunofluorescence

Adult rat or mouse testes were isolated and frozen in OCT in -80 °C hexane. Ten □m frozen sections were fixed in -20 °C acetone for 5 minutes. The sections were rinsed in 0.5 % Tween20/PBS and washed once for 10 min at room temperature in PBS containing 0.5% Tween20 with gentle shaking. Sections were incubated with MAbs or polyclonals to KLC-3, or KHC monoclonals (Clonetech) diluted in 1% BSA in PBS at 37 °C for 30 minutes in a humidified chamber. The sections were then washed 3X times in 1X PBS. Then fluorochrome conjugated secondary rabbit anti-mouse or goat anti rabbit antibody was added in 1%BSA in 1X PBS for 30 minutes at 37 °C in the humidified chamber. After final washes, sections were rinsed in H_2O, mounted onto glass slides and examined. Confocal immunofluorescence microscopic analysis was done in the University of Calgary Electron Microscopy and Imaging Facility.

Immunohistochemistry

Yeast two hybrid interactions

Yeast two hybrid interactions were tested as follows: KLC-3 and KLC-3 delta HR were cloned into pGAD424 previously in our lab (van der Hoorn, per coms). All genes and their derivative deletion mutants tested for interaction with KLC-3 were cloned in pGBT9 (both plasmids from Clonetech). Yeast strains SFY 526 and HF7c (Clonetech) were used to assay LacZ and His promoter activation respectively for the c-Mos/KLC and ODF-1/SPAG4 interactions. Yeast strain MaV 203 was also used to assay all these interactions. In all cases, positive controls and negative controls (empty vectors) were used. Yeast strains were co-transformed with pGBT9 and pGAD vectors and plated on -leu -trp plates to select for both plasmids. Most efficient transformation was achieved following Life-Technologies protocol: yeast strains were streaked on YPD plates and grown at 30C. Individual colonies were grown in a 5ml overnight culture (30C, 250rpm). The culture was diluted 1:20 and grown for 5-6hrs. Cells were pelleted and washed sequentially in distilled water, 1XTE/LiAcetate and then resuspended in 175ul TE/Lithium Acetate. 50ul of cells were added to 5ul freshly boiled/sonicated salmon sperm DNA (10mg/ml), 100ng of each plasmid, and 300ul of 1X PEG/LiAc. Cells were incubated at 30C for 30 min, then heat shocked for 30min at 42C, centrifuged for 6,000 rpm for 20-30s, supernatant removed and then resuspended in 300ul distilled sterile water. 100ul was plated on -leu-trp plate and grown at 30C for 2 days. Representative clones from each transformation were transfered to a master plate in triplicate and grown overnight. His promoter assay: the master plate was replica plated onto -his,-leu,-trp plates with 0mM, 5mM, 15mM and 25mM 3-AT and replica cleaned 3X. Plates were

grown overnight, and then replica cleaned 3X. Plates were then grown for several days and results tabulated. LacZ assay: Nitrocellulose filters were used to replica the master plate. These were immersed in liquid nitrogen (or alternatively frozen at -80C, thawed, frozen, thawed) and placed on blotting paper wet with Z-buffer/X-gal solution (Clonetech protocol) in a petri plate, and incubated at 30C overnight.

Generation of c-Mos deletion mutants

c-Mos deletion mutants were generated by long PCR using the Expand Long Template System from Boehringer Mannheim. Primers were: 3'primer for all reactions (cgg gaa ttc cgg cga tac ag), deletion 231(ccc gaa ttc tgc agc agc agc cc), deletion 330 (gcg gaa ttc gac tgg gga cag), 456 deletion (gca gaa ttc gca tcc caa cgg), 885 deletion (gcg gaa ttc ccg gag ctc ctg). Long PCR was performed according to the Boehringer Mannheim Long PCR protocol using the Buffer 3 system and annealing temperatures of 42C). The product was ethanol precipitated, digested with EcoR1, gel purified, self-ligated and used to transform competent XL1blue cells. Clones were analyzed by restriction analysis to verify the presence of the deletions.

KLC-3 deletions

The 1-726 deletion mutant was generated by cutting the full-length clone in pGAD 424 with SacI and SalI and BglII, blunt ending, self-ligating, transforming XL1blue and checking resultant clones by restiction analysis for the presence of the smaller fragment. TPR constructs were generated by PCR using degenerate primers spanning the TPR domains of all KLCs. 5'primer: ttt gaa ttc tac gag atc cca gc(a/t/c) (c/a)g, 3' primer gtc gac g(c/g)t gag gat ctc ctt gta. The primers have EcoR1 and SalI sites respectively: the PCR product was ethanol precipitated, digested with these enzymes, gel isolated and

ligated into pGAD424 cut with the same enzymes, transformed into XL1blue and clones screened by restriction enzyme analysis. Nt-KLC-3 (spanning 1-833) was generated previously in our lab (van der Hoorn, per coms).

Organisation of Results Section: in accordance with the hypothesis/research aims, the Results have been grouped together into three sections: I and II illustrate the experiments conducted to determine if KLC-3 is indeed a KLC family member, section III is aimed largely at an examination of the function of KLC-3 within the testis.

RESULTS

I: KLC-3 MAPS TO THE ERCC-2 LOCUS: HUMAN CHROMOSOME 19Q13.2

To investigate whether the rat cDNA was indeed the proposed KLC gene in the ERCC2 locus, the KLC-3 cDNA was used to probe a mouse genomic library at high stringency. If the genes are identical, genomic clones of KLC-3 should also harbor ERCC2 sequences, as the ERCC2 and the putative KLC gene are linked tail to tail with a 196 bp separation between their 3' ends. Resulting clones were therefore analyzed by PCR for the presence of KLC-3 and ERCC2 sequences. PCR primers were designed to amplify the last introns of KLC-3 (between exons 10 and 11) and ERCC2 (between exons 19 and 20) (Fig. 4), whose sequences were based on KLC-3 cDNA and on the published ERCC2 genomic DNA (Lamerdin et al., 1996), respectively. To verify the close proximity of the two genes, PCR using primers that spanned the 3' exons, introns and intergenic space of these two genes was also performed. In all PCR reactions high molecular weight mouse genomic DNA and cloned KLC-3 genomic DNA were compared. The results shown in Figure 4A demonstrate that i) isolated genomic DNA and cloned genomic DNA generate identical PCR fragments for all primer combinations, (compare lanes 1 and 2, 3 and 4, 5 and 6), ii) cloned genomic KLC-3 DNA generates

expected PCR products with both ERCC2 primers (lane 1) and KLC-3 primers (lane 5) and iii) ERCC2 and KLC-3 are linked as predicted (lanes 5 and 6).

To verify these results, the respective PCR products were cut with restriction enzymes, which were predicted based on the published sequence. The PCR products of ERCC2, KLC-3 and the spanning sequences were cut with Apa 1, Hpa 1 and Bgl II respectively. The results in Figure 1B show original PCR products (lanes 1&2, 5&6, 9&10) juxtaposed with the products of the digestions (lanes 3&4, 7&8, 1d1&12 respectively) and demonstrate that the bands were cut as predicted. (Note that genomic PCR product of KLC-3 primers did cut, albeit poorly. Technical problems are suspected). It is concluded that the putative KLC gene in the ERCC2 locus and KLC-3 are identical. Thus, ERCC2 and KLC-3 are linked tail to tail, and KLC-3 localizes to genomic locus 19q13.2 in humans.

The results demonstrate that the rat cDNA is identical to the putative KLC gene in the mouse ERCC2-locus. Based on the conservation of this locus in hamster, mouse and human, it is most likely also conserved in the rat. In order to verify this prediction, a final PCR experiment wasconducted. Genomic DNA isolated from rat and mouse was subjected to PCR analysis using the primers spanning ERCC2 and KLC sequences, and the products compared by electrophoresis. As shown in figure 1C, the products of rat DNA (lane 1) and mouse DNA (lane 2) PCR reactions are identical in size, and match predicted sizes. It is concluded that the ERCC2 locus sequences are conserved in the rat.

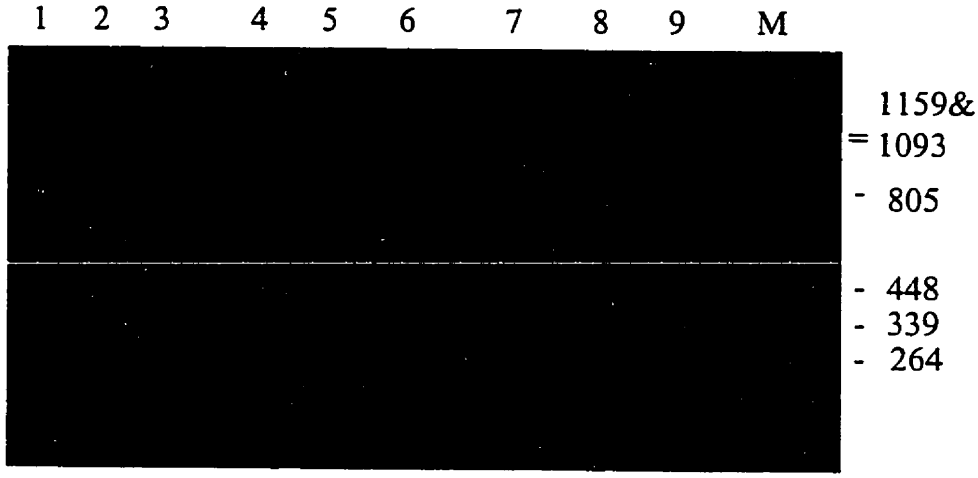
Figure 4

A) To establish that KLC-3 and ERCC2 sequences are linked as predicted, a phage library was screened with KCL-3 probes. Resulting clones were compared by PCR analysis to genomic DNA or no DNA control. Primers were made based on published sequences, and targeted to both KCL-3 and ERCC2. Lanes 1,4,7:cloned phage DNA, lanes 2,5,8: mouse genomic DNA, lanes 3,6,9: no DNA control. Lanes 1-3 sample amplified with KLC-3 specific primers (predicted size 300bp), 4-6: amplified with ERCC2 specific primers (predicted size 274bp), 7-9:amplified with primers specific for both and thus spanning the intragenic region (predicted size 813bp). The identity of the genomic and cloned DNA and match to predicted band size indicates that KLC-3 and ERCC2 are linked as predicted

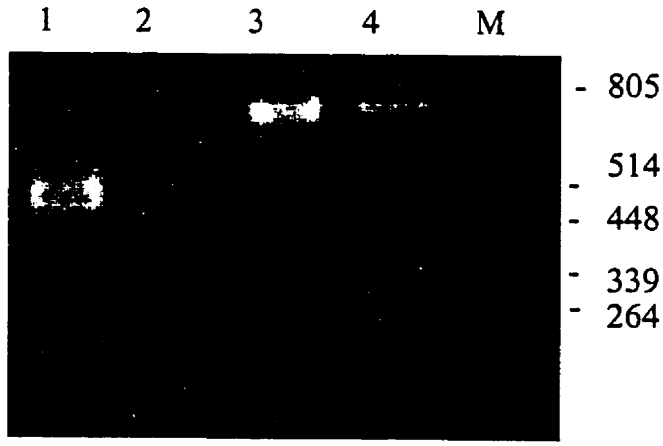
B) To confirm the identity of these bands, the intragenic PCR product (lanes 3&4) was digested with BglII, predicted to cut once within this region. Note the identity of the restriction digest pattern for both genomic and cloned DNA (lanes 1&2).

C) Rat and mouse genomic DNA was compared by PCR analysis to determine if ERCC2 and KLC-3 are linked in the rat as they are in the mouse. Genomic DNA was used as a template for PCR with primers spanning ERCC2 and KLC-3 sequences as described above (lanes 1,2) Both samples generate a product of identical size, predicted to be 813 bp. Lanes, from left to right (M, M: Lambda and pBluescript ladders, 1- mouse DNA as a template, 2 – rat DNA as a template).

A


1 2 3 4 5 6 7 8 9 M
1159&
= 1093
- 805
- 448
- 339
- 264

B


1 2 3 4 M
- 805
514
-
- 448
- 339
- 264

C


M M 1 2

II: KLC-3 IS A NOVEL MEMBER OF THE KLC FAMILY

II.a: Sequence Analysis reveals that KLC-3 is a novel but divergent member of the KLC family

Previous Blast searches and sequence alignments in our laboratory suggested KLC-3 was a KLC (van der Hoorn, per coms). Furthermore, previous groups had assigned this putative gene to the KLC family based on sequence homology (Lamerdin et al., 1996)(Rahman et al., 1998). However, in all cases the analysis was not rigorous and did not define regions of high conservation and the presence or absence of the signature KLC motifs as defined in the introduction (sect. II.a). Therefore, the sequence of KLC-3 was analyzed in more detail in an effort to gain insight into the evolution of the gene, the structure of the protein, and its assignment to the KLC family.

Iinitially, multiple KLC protein sequences were obtained from the NCBI database and aligned with the predicted protein sequence of KLC-3 by OMIGA. Results are shown in Figure 5. Figure 5A reveals that KLC-3 fits the general KLC sequence homology patterns: high sequence identity in the putative Heptad Repeat (HR) and tetratricopeptide repeat (TPR) regions, and little conservation elsewhere. Overall KLC-3 sequence homology to other KLCs ranges around 50%. Collectively, this is good evidence that KLC-3 is a KLC. The pair wise alignment identities and the dendogram (Fig. 5B and C) hint at the origins of KLC-3. The dendogram predicts that lower eukaryotes such as *C. elegans*, *Drosophila*, squid and sea urchin have only one KLC. Presumably gene duplication in higher eukaryotes has generated the additional KLC-2 and 3 genes, which are derived from the same ancestral gene (KLC-1) and have

subsequently diverged from each other and KLC-1 in sequence. It appears that all KLCs except KLC-2 and KLC-3 are homologues

of rKLC-1. KLC-2 and 3 sequences are more divergent as they are separate genes, and KLC-3 has less sequence conservation than KLC-2. It is concluded that KLC-3 is likely a novel KLC gene, likely not represented in lower eukaryotes, and the least conserved KLC. These conclusions are consistent with published observations. (Some inaccuracies regarding KLC sequence conservation are evident in the pair wise identities and dendograms. For example, hamster KLC is further removed from mouse and rat than the chicken sequence is. This is most likely a consequence of the fact that different splice isoforms are represented). The KLC-3 sequence was additionally analyzed by OMIGA for phosphorylation sites and protein motifs, revealing several PKC sites, a leucine zipper and identifying the TPR region as a "KLC motif" (5D). These are commented on in more detail below, where the sequence has been analyzed piecemeal in more detail by sequence comparisons with KLC-2 and two KLC-1 homologues (mouse KLC-1 and rat KLC-1)

i)KLC-3 does not have multiple start Methionines: All KLCs except for sea urchin have multiple methionines present within the first 30 amino acids (data not shown). The analogous region in KLC-3 has only one methionine. KLC-3 does, however have multiple valines in this stretch, which in other proteins have been suggested to act as alternative initiation sites (Nagao, 1995b). In vitro transcription and translation reactions (tnt) reliably generate multiple KLC-3 bands suggesting either proteolysis or alternative start sites (refer to Figs 8A and B, showing multiple bands for KLC-3 translations).

Figure 5

KLC protein sequences from various organisms were aligned using OMIGA gene analysis program, which generates a figure (A), a pair wise identity table (B), and a dendogram (C). A) The figure is color coded to highlight mutation hotspots (red), sequence identities (dark blue), and sequence conservation (light blue and green). The color-coding scheme of OMIGA used here calculates the deviation of individual residues from the mutational frequency of all residues at that position. Note that KLC-3 fits the general pattern of high sequence conservation in the HR domain (aprox. 50-150), higher conservation in the TPR domains (aprox. 200-450), and no sequence conservation in the N-terminus, C-terminus and PEST (150-200) regions. However, KLC-3 sequence does have the highest mutational frequency in the conserved TPR and HR regions. B) The pair wise alignments identities of the KLC sequences. KLC-3 sequence is most similar to mouse KLC-2, and is less similar to mouse KLC-1 than the KLC of any other organism. C) The dendogram suggests that all KLC genes except KLC-2 and KLC-3 are homologues, and that these two genes are present in organisms of complexity equal to or higher than chicken. D) Protein motif searches of the KLC3 cDNA sequence using the OMIGA phosphorylation sites and selected protein motif parameters.

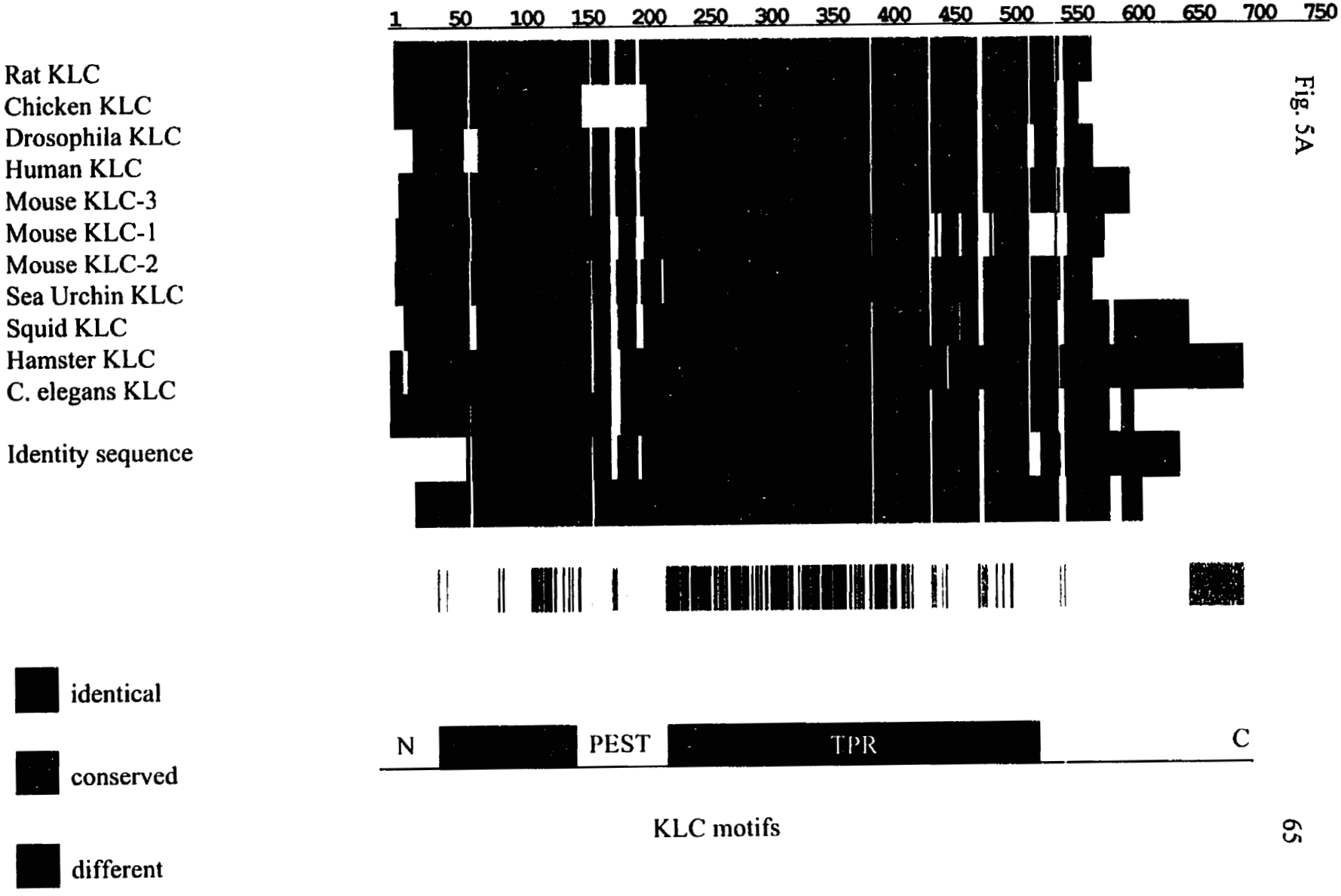

1 50 100 150 200 250 300 350 400 450 500 550 600 650 700 750
Rat KLC
Chicken KLC
Drosophila KLC
Human KLC
Mouse KLC-3
Mouse KLC-1
Mouse KLC-2
Sea Urchin KLC
Squid KLC
Hamster KLC
C. elegans KLC
Identity sequence
identical
conserved
different
N
PEST
TPR
C
KLC motifs

Fig. 5A

Figure 5B

Key to sequence names:

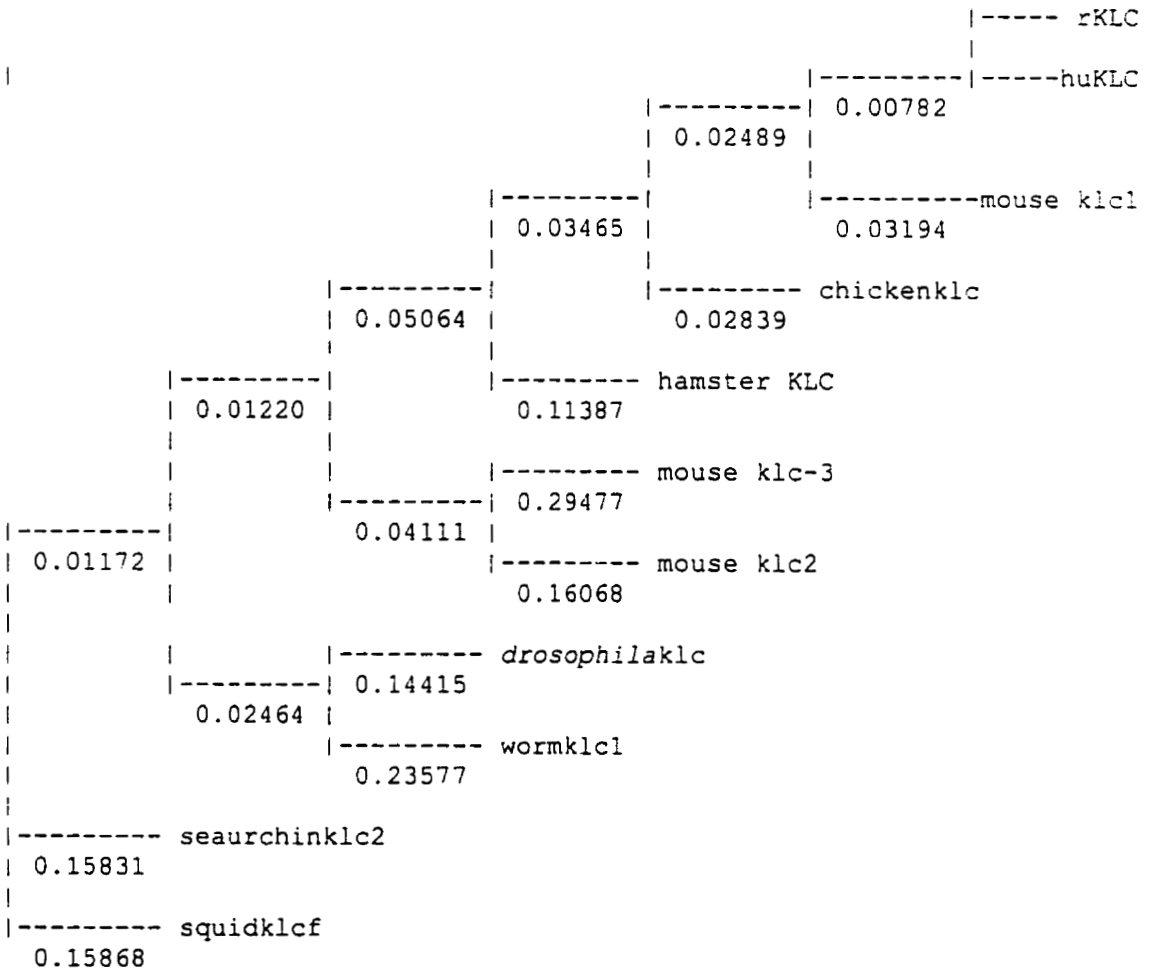
Sequence 1: rat klc-1
Sequence 2: chicken klc
Sequence 3: *Drosophila* klc
Sequence 4: human klc
Sequence 5: mouse klc-3
Sequence 6: mouse klc-1
Sequence 7: mouse klc-2
Sequence 8: sea urchin klc
Sequence 9: squid klc
Sequence 10: Translation of hamster klc
Sequence 11: *C. elegans* klc

Pair wise alignment percentage identities

	1	2	3	4	5	6	7	8	9	10	11
1		92	68	97	**48**	95	67	69	70	84	58
2			68	93	**47**	90	72	72	73	79	63
3				68	**45**	66	64	65	68	64	62
4					**51**	94	65	66	67	85	55
5						**49**	**54**	**51**	**46**	**49**	**44**
6							65	68	69	81	56
7								59	59	67	52
8									68	66	55
9										63	56
10											53
11											

Figure 5C

Dendrogram



* Distance = 1 – (%identity/100%)

Fig 5d

CK2 Phospo site
2 PKC Phospo sites
2 PKC Phospho sites
Leu zipper
KLC motif
Amino acid #
0
100
300
500

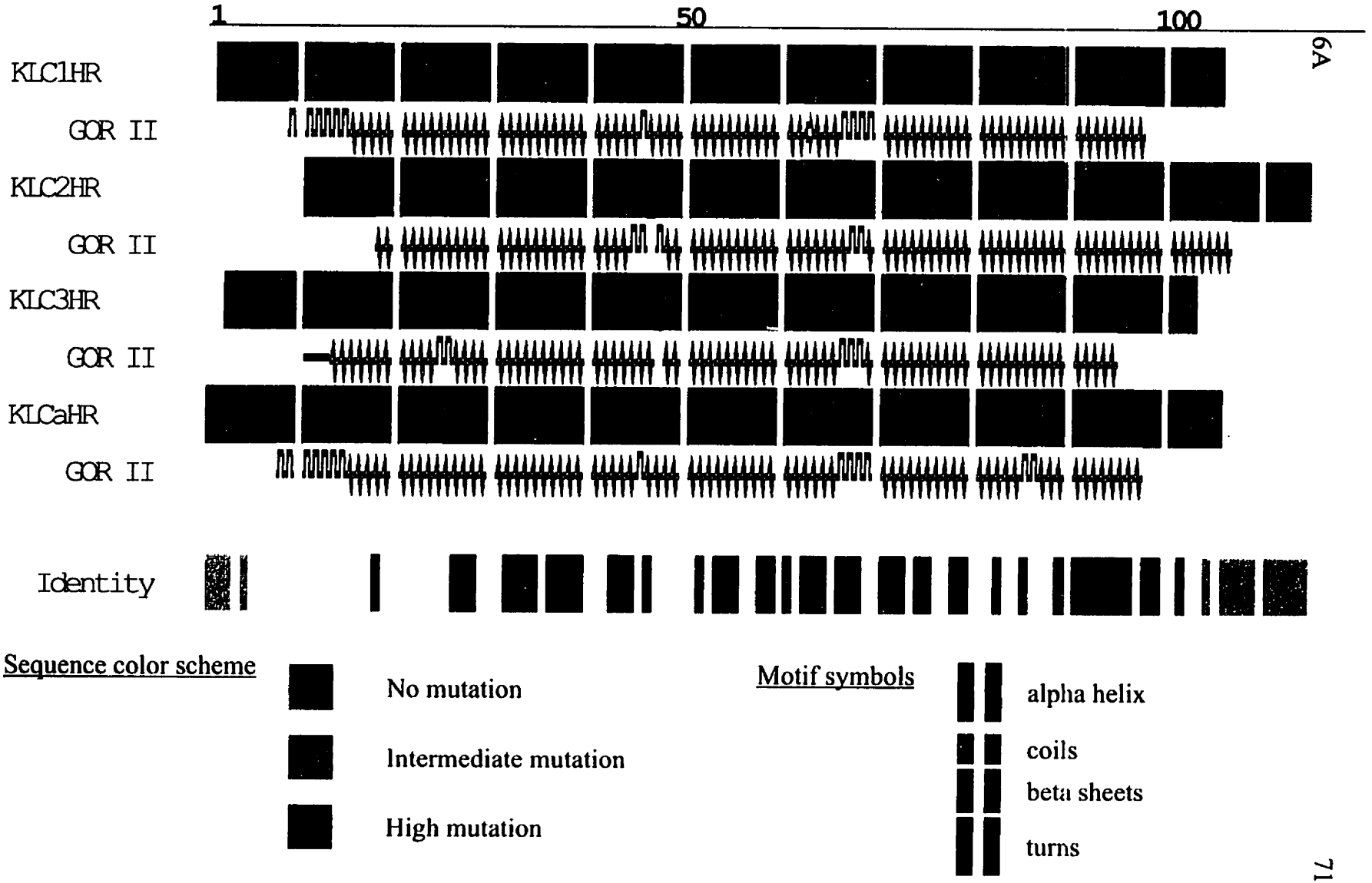
conclude that KLC-3 may have alternate initiation sites, but does not fit the criteria of multiple start methionines.

ii) KLC-3 has Heptad Repeats: To ascertain the sequence conservation specific to this region, the region corresponding to the HR of mKLC-1, 2 and rKLC-1 (amino acids 49-155, 48-154, 37-145 respectively) were aligned to the corresponding region of high homology in KLC-3 using OMIGA. Both Chou & Fassman and Gor II algorithms were used to predict secondary proteins structures for these sequences. The following conclusions are possible: KLC-3 sequence identity is higher in this region (aprox. 55%) than for full-length alignments (aprox 50%) (compare 6B with 7B), indicating evolutionary conservation of this region. However, KLC-3 is less conserved relative to rKLC-1 than mKLC-2 is. Nonetheless, the predicted secondary structure is almost identical for all four genes, and consists entirely of helices interrupted by turns, the spacing of which is similar for the four genes (Fig 6A). OMIGA predicts that all four proteins have a leucine zipper motif at amino acids 90-110 (Fig. 5D), but curiously this has never been reported. The dendogram (6C) shows, and sequence identities indicate, that KLC-2 and 3 sequences in this stretch have diverged from KLC-1, KLC-3 more so. Based on the conservation of secondary structure and the high sequence identity, it is concluded this region likely mediates KHC binding for KLC-3. The sequence divergence in this region by KLC-3 was not investigated, but could well represent functional differences such as self-association (see discussion).

iii) KLC-3 has a PEST region: The PEST region is defined in KLCs largely by high content of the amino acids shown in table 1, low sequence conservation in this area, and

Figure 6

Analysis of mouse KLC-1, 2,3 and rat KLC-1 (shown as KLCa) Heptad Repeat Domain Sequences. The HR regions for KLC-1, 2 and rat KLCa were based upon published results (Cyr et al., 1991; Rahman et al., 1998). The HR region of KLC-3 was identified by alignment of KLC-3 with other KLCs (figure 5). The alignments, predicted secondary structures, pair wise alignments and dendogram of the HR domain sequences were done using OMIGA gene analysis program. A) Alignment of KLC HR domains, color-coded for identity and mutation hotspots as in figure 5A. Note the high sequence identity amongst all four genes, with KLC-3 showing the highest mutation rate (red). The predicted secondary structure, as calculated using the GOR II algorithm, is almost identical for KLC-1, 2, a, and KLC-3. B) Pair wise alignment identities for these KLC genes: the homology is highest between mouse KLC-1 and rat KLCa. KLC-3 sequence is the least conserved of the four genes and most similar to KLC-2. C) Dendogram of HR sequences suggests the evolutionary divergence of KLC-2 and 3 sequences from mouse KLC-1 and it's homologue rat KLCa.


1
50
100
KLC1HR
GOR II
KLC2HR
GOR II
KLC3HR
GOR II
KLCaHR
GOR II
Identity
Sequence color scheme
No mutation
Intermediate mutation
High mutation
Motif symbols
alpha helix
coils
beta sheets
turns

6A

71

Figure 6B

Key to sequence names:

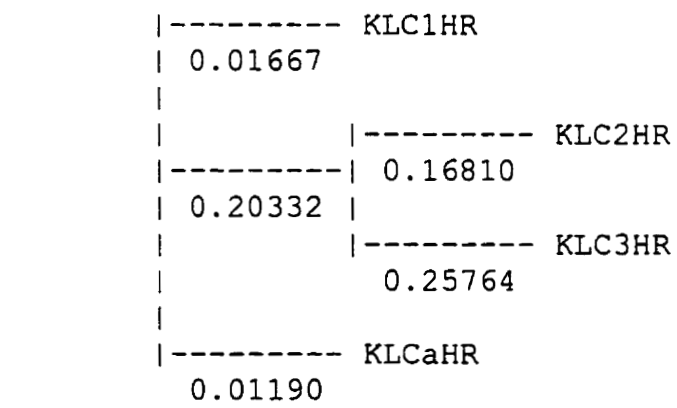
Sequence 1: KLC1HR
Sequence 2: KLC2HR
Sequence 3: KLC3HR
Sequence 4: KLCaHR

Pair wise alignment percentage identities

	1	2	**3**	4
1		60	**52**	97
2			**57**	61
3				**52**
4				

Figure 6C

Dendrogram



* Distance: 1 –(%identity/100%)

	Proline		Serine		Threonine		Aspartate		Glutamate		Total %
KLC-3	7	14%	5	10%	0	0%	3	6%	7	14%	44%
rKLC-1	4	8%	7	14%	0	0%	11	22%	3	6%	50%

TABLE 1: Results of PEST sequence analysis by OMIGA. PEST sequences defined as those between the HR and TPR sequences. The boundaries of these sequences have been published for KLC-1, and were deduced for KLC-3 by sequence alignment with various KLCs (fig 5A).

by sensitivity of this region to proteolytic cleavage. The results, as tabulated by OMIGA, show that KLC-3 fits the criteria of having a high percentage of the requisite amino acids, (44% as compared with 48% in the original citation (Beushausen et al., 1993) and 50% for rat rKLC-1). The sequence conservation between KLC-3 and rat rKLC-1 is only 18% as defined by OMIGA (vs. 13% in original citation). Based on this it is concluded the PEST region is present in KLC-3.

iv) KLC-3 has a modified TPR domain: To ascertain sequence conservation of KLC-3 in this domain, sequences representing the TPR domain of rat rKLC-1, mKLC-1 and KLC-2 and were aligned using OMIGA (204-413 + 456-497, 202-412 + 455-495, and 188-388 + 438-479 respectively). The sequences of the TPR domains have been published for KLC-1 and 2(Rahman et al., 1998). Additionally, the sequences of the individual TPR repeats (1 through 6) have also been reported (Gindhart, Jr. and Goldstein, 1996), and these were individually aligned with KLC-3 and mKLC-1. The results shown in the alignments, pair wise sequence identities and dendogram (fig 7A, B and C) are similar to those presented for the HR region: KLC-3 is the most divergent KLC in the TPR sequence, and KLC-2 and 3 have both diverged in TPR sequences from the mKLC-1 genes. The sequence identity is highest in the TPR region for all KLC genes, including KLC-3: identity to mKLC-1, 2 and rKLC-1 ranges around 76% here, as compared to aprox. 55% in the HR, 32% in the N-terminus, and 18% in the PEST region. The predicted secondary structure in this region is also strikingly conserved: note the high alpha helix content and the conserved helix-breaking turns (7A). The individual TPR motifs are highly conserved amongst the KLCs. Repeats 1-5 of KLC-3 conserve approximately one-half of the putative TPR motif amino acids (7D), in line with similar

observations with other KLC TPRs. Of interest is the absence of the 6th TPR repeat in KLC-3: the corresponding sequence in this region of KLC-3 as assigned by OMIGA displays no sequence homology to the 6th TPR repeat, and is not represented elsewhere in the protein sequence. Based on the high sequence identity and predicted protein structure similarity to KLC family members, it is concluded that this region of KLC-3 likely serves a similar function as it does in other KLCs (putatively in cargo binding). The lack of a 6th TPR and the presence of sequence divergence suggest unique/subtle twists to TPR function in KLC-3.

v) KLC-3 has divergent C-terminal sequences: The C-terminus of KLC-3 is short relative to other KLC sequences (22 amino acids) and shows no sequence homology to other C-terminal sequences. Also noteworthy is the sequence gap for KLC-3 at amino acids 400-450 (fig 5A). The absence of this sequence is reminiscent/suggestive of alternative splicing mechanisms. Similar deletions have been reported for other KLC splice isoforms. The possibility of alternative splicing mechanisms was not investigated. It is conclude that the divergent C-terminus of KLC-3 is consistent with KLC paradigms, and suggests mechanisms whereby KLC functional diversity is generated.

Figure 7

An alignment of TPR sequences was generated by OMIGA gene analysis program. TPR sequences of mouse KLC-1, 2 and rat KLCa were based on published results(Rahman et al., 1998; Cyr et al., 1991). The corresponding TPR repeats of KLC-3 were identified by alignment with the individual TPR repeats 1 through 6 from *Drosophila* (Gindhart, Jr. and Goldstein, 1996). A) Figure of TPR sequences color-coded as in Fig. 5. Note the high sequence identity and the divergence of KLC-3 from this at various residues absence of the 6^{th} TPR repeat in KLC-3. The GOR II algorithm predicts that all KLCs share a very similar protein structure in the TPR region, composed largely of helices (red) interrupted by turns (black). B) Pair wise alignment identities of the respective TPR domains. Compare the sequence conservation of KLC-3 here (aprox.76%) to the overall conservation (aprox. 50%, fig. 5B). C) The dendogram again suggests evolutionary divergence of KLC-2 and 3 sequences from mouse KLC-1 and rat KLCa. D) Alignment of KLC-3 TPR repeats 1-5 with the 8 amino acids which are loosely conserved in TPR motifs (6 repeats are shown for the consensus TPR to highlight the absence of the 6^{th} TPR in KLC-3).

1
50
100
150
200
250
KLC-1TPR
GOR II
KLC-2TPR
GOR II
KLC-3TPR
GOR II
KLCaTPR
GOR II
Identity
1
43
85
127
168
210
252
TPR1
TPR2
TPR3
TPR4
TPR5
TPR6
Sequence color scheme
No mutation
Intermediate mutation
High mutation
Motif symbols
alpha helix
coils
beta sheets
turns

7A

Fig. 7B

Key to sequence names:

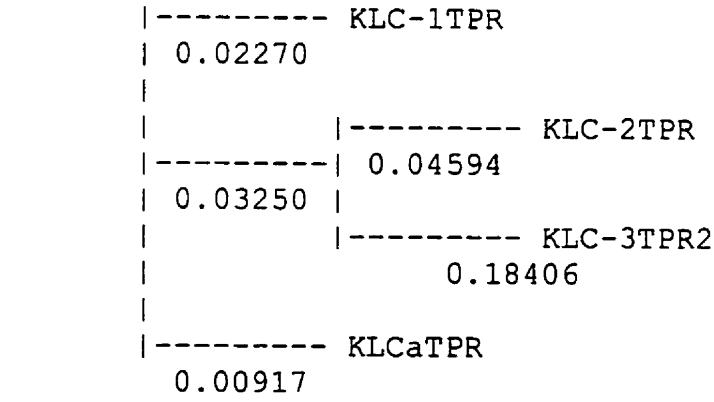
Sequence 1: KLC-1TPR
Sequence 2: KLC-2TPR
Sequence 3: KLC-3TPR
Sequence 4: KLCaTPR

Pair wise alignment percentage identities

	1	2	3	4
1		89	**76**	96
2			**77**	91
3				**77**

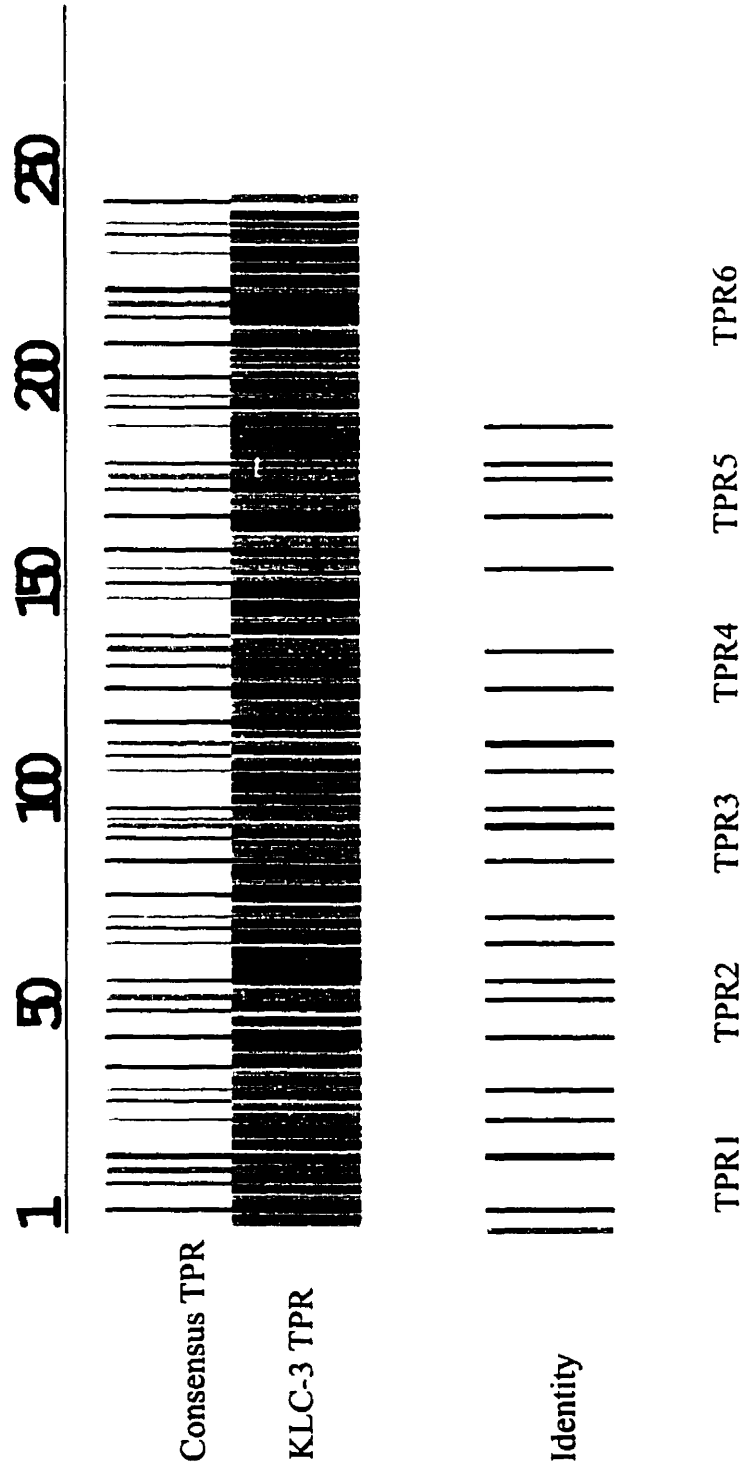
Fig. 7C

Dendrogram



*Distances: 1 – (%identity/100%)

7D



Color code is set to amino acid type, not mutation frequency

vi) KLC-3 has some of the conserved KLC phosphorylation sites: Conserved PKA, PKC and Tyrosine Kinase sites have been reported for KLC family members. OMIGA was used to search KLC-3 sequences for PKC, Tyrosine Kinase sites and PKA motifs. Multiple PKC sites were detected in mKLC-1, rKLC-1, KLC-2 and KLC-3. The sites are present in two clusters: one in the HR region, and the second in the TPR/C-terminus of the TPR region. Both of these clusters were conserved in all four KLCs, with some variability. mKLC-1, KLC-2 and rKLC-1 additionally had several Tyrosine Kinase sites not represented in KLC-3.

vii) Summary: KLC-3 has KLC family signature motifs, including the HR, PEST, TPR, divergent C-terminus and PKC phosphorylation sites. However, KLC-3 consistently displays subtle deviations from KLC family members: only one start methionine, the shortest C-terminus, absence of the 6^{th} TPR repeat and absence of Tyrosine Kinase phosphorylation sites and the lowest sequence conservation. Based on this, it is concluded that KLC-3 is a novel member of the KLC family. Further, it is hypothesized that the sequence divergence in KLCs represents functional specialization of this KLC gene.

II.b: KLC-3 Interacts with KHC *in vitro* via the Heptad Repeat Region

By definition, KLCs interact with KHC subunits. The high sequence homology of KLC-3 to KLCs strongly suggests that it is a KLC family member, but definite proof requires a functional demonstration: namely that KLC-3 interacts with KHC. In order to verify this fact, immunoprecipitations were done using in vitro translated KLC-3 and Kif5c. KLC-3 and brain KHC isoform Kif5c were translated individually or were co-carried out using anti-KLC-3 mAb, and precipitated proteins were analyzed by SDS-

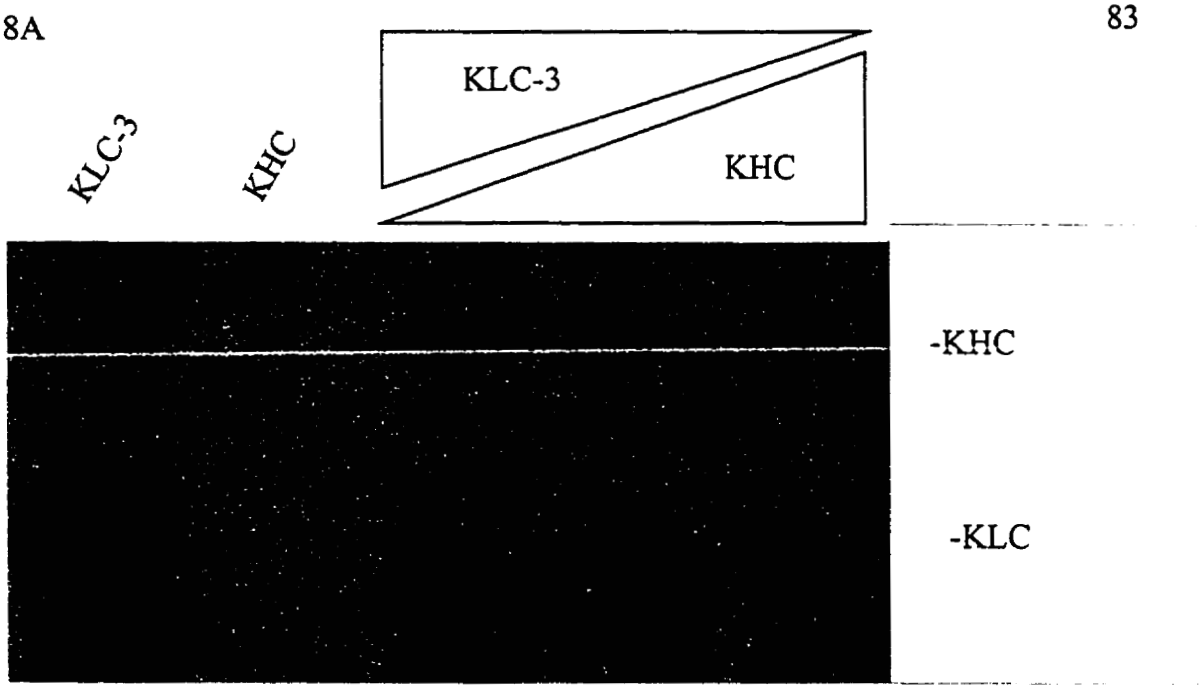
PAGE. The results, shown in Fig. 8A, demonstrate that Kif5c efficiently associates with KLC-3 upon co-translation. Neither Kif5c by itself, nor an unrelated molecule translated together with KLC-3 in the same fashion (SPAG4, Fig. 19) were found to translated (S^{35} labeled) using different ratios of KLC-3:KHC. Immunoprecipitations were immunoprecipitate with KLC-3. It is concluded that KLC-3 can associate with Kif5c.

To show that this association is specific, a deletion mutant of KLC-3 lacking the Heptad Repeat region was generated. Deletion of this region in other KLC isoforms abolishes binding to KHC. The immunoprecipitations were repeated as above, with the addition of KLC-1 as a positive control. The results in Fig 8B show that both wild-type KLC-3 and KLC-1 immunoprecipitate Kif5c, whereas the HR deletion mutant does not. It is concluded that KLC-3 can form a complex with Kif5c, and that this is likely mediated by the HR motif of KLC-3. These results are consistent with the predictions from sequence analysis presented above.

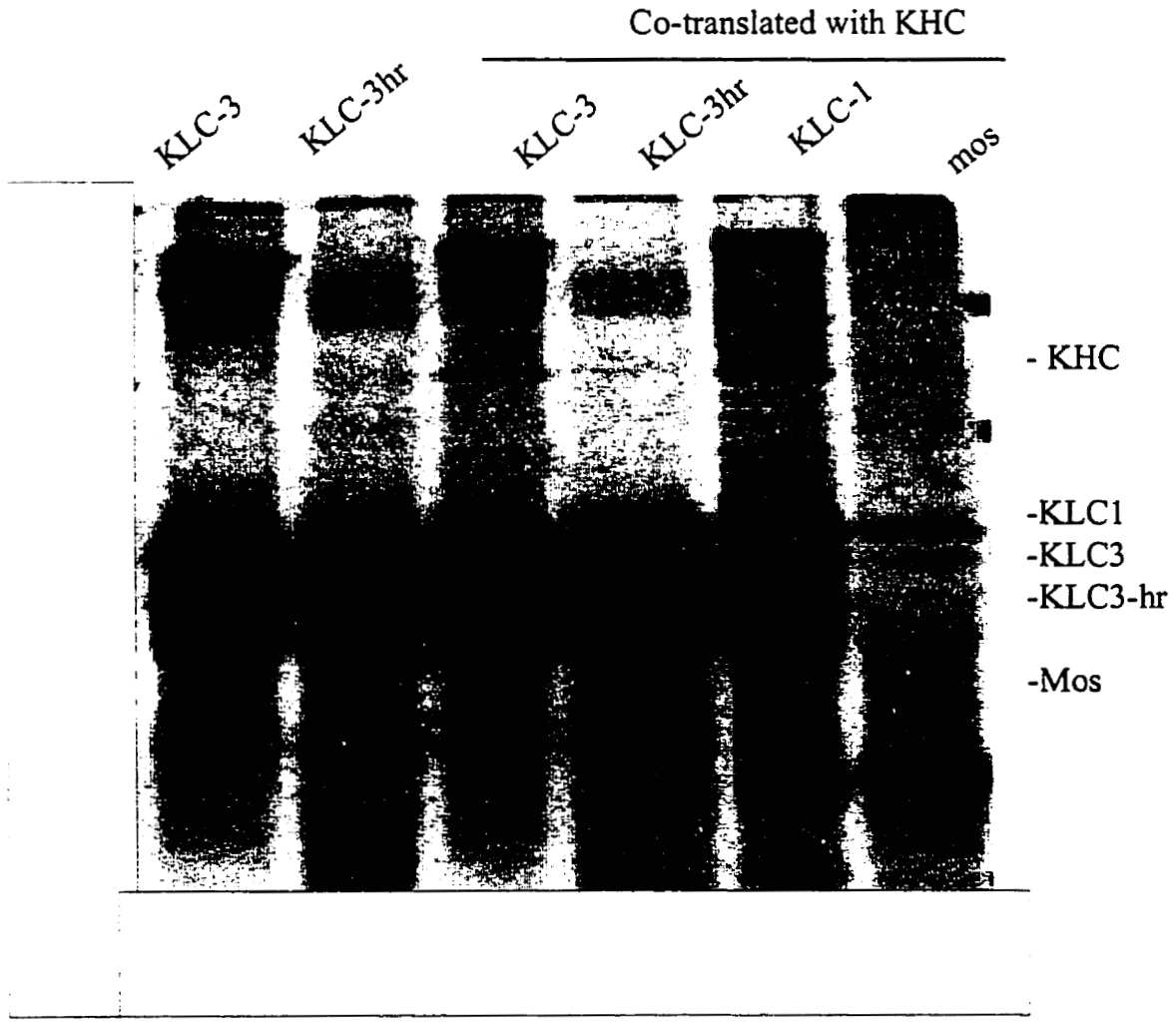
Figure 8

A) In order to determine whether KLC3 interacts with KHC, co-immunoprecipitations of KLC-3 and Kif5c were conducted. Both proteins were transcribed and translated in vitro from a plasmid template in the presence of radioactive label, either individually (first two lanes), or together at different ratios (last three lanes). This was followed by immunoprecipitation with antibodies targeted to KLC-3. The KHC derived band immunoprecipitates only in the presence of KLC-3. Increasing the amount of KHC template (last three lanes) increases the intensity of this band, suggesting that KHC levels are the limiting factor. B) The HR region of KLC-3 was deleted by a mutagenesis technique, and this construct was used in a similar experiment as above. The mobility of KLC-3hr mutant is faster than full length KLC-3, consistent with its smaller size (first two lanes). When translated and immunoprecipitated in the presence of Kif5c, only full-length KLC-3 brings down Kif5c. Full-length KLC-1 also immunoprecipitates Kif5c (positive control), whereas c-Mos (the negative control) does not.

8A


KLC-3
KHC
KLC-3
KHC
-KHC
-KLC

B


Co-translated with KHC
KLC-3
KLC-3hr
KLC-3
KLC-3hr
KLC-1
mos
- KHC
-KLC1
-KLC3
-KLC3-hr
-Mos

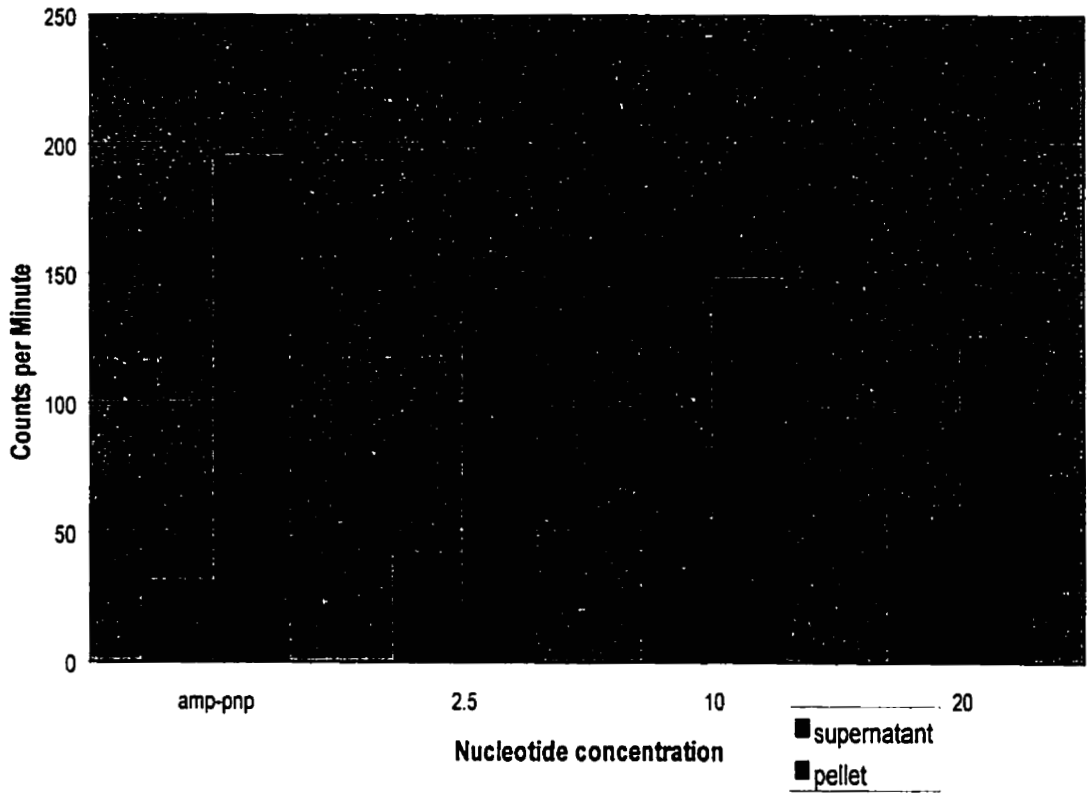
II.c: KLC-3 is stabilized on MTs by AMP-PNP and released by ATP:

The stabilization of kinesin on microtubules by the non-hydrolysable ATP analogue AMP-PNP and its subsequent release by ATP is a distinguishing characteristic of kinesin. Therefore, this assay was conducted in order to further verify our classification of KLC-3 into the KLC family. Taxol stabilized MTs were isolated from brain and testes in the presence of AMP-PNP, thus ensuring kinesin co-purification. These MTs were incubated with in vitro translated, ^{35}S-radiolabeled KLC-3 (in the presence of AMP-PNP) and then pelleted. The MT pellet was then subjected to three rounds of washing and pelletting. Washes were done with AMP-PNP, 2.5, 10 or 20mMATP. Aliquots of both the MT pellet and the supernatant were analyzed by SDS-PAGE/autoradiography, the KLC-3 bands identified and counted in an LSC. The results are shown in Fig.9. Note that KLC-3 binding to MTs was diminished in the presence of ATP and this effect is mirrored by an increase in KLC-3 partitioning to the soluble fraction. Furthermore, this effect increases with ATP concentration. It is concluded that KLC-3 is stabilized on microtubules in the presence of AMP-PNP and is solubilised by ATP. This is consistent with previous observations of kinesin binding dynamics to MTs. Since KLC-3 can form a stable complex with KHC, it is concluded that KLC-3 likely forms a complex with KHC that is capable of binding MTs.

Figure 9

In vitro translated, radiolabelled KLC-3 was incubated with purified and taxol stabilized microtubules in the presence of either AMP-PNP, and then subjected to a microtubule pelleting and wash sequence in the presence of either AMP-PNP or increasing amounts of ATP. Aliquots of both the supernatant and pellet were analyzed by SDS-PAGE. The KLC-3 derived bands were identified by autoradiography. These were excised from the gel and counted in a Beckmann Liquid Scintillation Counter (counts per minute). The bars shown represent the amount of KLC-3 derived radioactivity in either the supernatant or the pellet under the various conditions (AMP-PNP, or various concentrations of ATP). As is evident from the graph, supplanting AMP-PNP (first set of bars) with ATP (last three sets of bars) induced an increase in KLC-3 partitioning to the supernatant and a corresponding decrease in partitioning to the MT pellet.

9


250
200
150
100
50
0
Counts per Minute
amp-pnp
2.5
10
20
Nucleotide concentration
supernatant
pellet

III: KLC-3 MAY HAVE A NOVEL FUNCTION IN VIVO

III.a: Blast Search Supports the Hypothesis of Functional Diversity and Reveals KLC-3 expression in extra-testicular Tissues

The gene duplication and sequence divergence of KLC genes in higher eukaryotes supports the hypothesis of functional diversity. One KLC gene has been cloned from lower eukaryotes, 3 from higher eukaryotes. Subsequent to the publication of *C. elegans* and *Drosophila* KLC genes, these genomes have been completely sequenced and published. BLAST searches were therefore conducted to test the hypothesis that higher eukaryotes require additional KLC genes as a consequence of their increased complexity. KLC-3 sequences were used as a query. The results are summarized in table 2. In line with previous predictions, fungi express no KLC genes, lower eukaryotes encode only one, while mammals (and potentially birds) encode at least three. The possibility of fourth KLC gene in humans is hinted at by the presence of a short but highly homologous sequence on human chromosome 6. For the human genome queries, KLC-1, 2 and 3 sequences were used as "positive controls". The genomic loci of these genes produced matching sequence lengths varying from 60 to 170 bp in length, ranging from 80 to 90% identity to KLC-3. This is in the range of the results obtained for the sequence on human chromosome 6. Thus this sequence may represent a 4^{th} KLC gene, although it could also be a completely unrelated gene. Regardless, the results confirm previous predictions and lend support to the hypothesis of KLC functional diversity.

Also interesting was the fact that KLC-3 sequences were detected in rat eye lens and various mouse tissues. This is in contrast to initial results from our lab, based on

Organism	Results of query	Matching sequence length	% Identity	Tissue/ chromosome
E. coli complete genome	no KLC expressed	---	---	---
Yeast complete genome	no KLC expressed	---	---	---
C. elegans complete genome	only 1 KLC	---	---	---
Drosophila complete genome	only 1 KLC	---	---	---
Mouse EST*	potential KLC-3 mRNA	376 nt 384 nt 339 nt 93 nt	96% 95% 89% 97%	embryo carcinoma, adult placenta, mammary gland, 7.5 dpc embryo
Rat	KLC-3 mRNA	2 clones, aprox 100nts	98 - 100%	rat eye lens(Bonaldo et al., 1996)
Human Genome incomplete genome, non-contiguous sequences	potential for a 4th KLC gene	55bp	85%	human chromosome 6 p12.3-21.2

TABLE 2: Results of BLAST searches using KLC-3 query sequences. Matches were determined subjectively based on hit length, identity, expect values and localization of matches within the KLC-3 gene sequence. *Many mouse EST matches were omitted due to space constraints.

Northern blots, suggesting that KLC-3 is testis specific. The KLC-3 homologous sequences from mouse EST's present a curious puzzle: the homology is too high to represent KLC-1 or 2, but in some cases potentially too low to be the mouse homologue of KLC-3. It is possible that sequencing errors, artifacts or alternative splicing events explain the lack of perfect sequence identity. It is concluded from these results that KLC-3 may be expressed in a wide range of rodent tissues and in various developmental stages.

III.b: RT-PCR demonstrates that KLC-3 has a unique expression pattern in testis

The BLAST searches presented above demonstrate KLC-3 expression in various tissues. However, previous results from our laboratory suggested spermatid specific expression of KLC-3. Furthermore, I have proposed the testis as a good model system for the study of kinesin expression and function. In order to test these observations/predictions, RT-PCR analysis of kinesin genes was conducted. mRNA was isolated from various mouse tissues and additionally from premeiotic pachytene spermatocytes and round spermatids, quantified and equal amounts used for cDNA generation using random hexamer primers. The cDNA was initially characterized for RT-PCR suitability with actin specific primers. As shown in Fig 10A, RT-PCR generated a band of the predicted size, and the intensity of this band was equivalent for all samples. This indicated that the cDNA was suitable for analysis, and no gross differences in quantitation had occurred amongst samples.

i) KLC-3 expression is highest in spermatids: Next, two different sets of KLC-3 specific primers were used to analyze KLC-3 expression. Cloned KLC-3 DNA was used as a control in both cases, and produced bands of identical size .The results obtained with

one set of primers are shown in figure 10B. KLC-3 was detected in all tissues analyzed, however the intensity of the bands varied considerably amongst these tissues, barely detectable in liver, high in the testis and highest in round spermatids. The identity of the second (smaller) band in the spermatocyte lane is unknown, but is not thought to represent KLC-3. Although PCR is generally not interpreted as quantitative, several lines of evidence suggest that the band intensities could reflect levels of gene expression. First, a northern blot probed with KLC-3 and actin probes showed constant actin levels, but high KLC-3 signal in spermatids, a weaker signal in total testis, and no signal in spermatocytes (van der Hoorn, per coms). This suggests that mRNA levels were below the detection limit of Northern analysis for all samples except spermatids and testes, which are also the brightest bands from the RT-PCR. Second, the band intensities of other kinesin genes (and ERCC2) (Fig 10) also vary, in a similar but distinct manner from KLC-3 (especially in the germ cell fractions). However actin band intensities did not vary. This suggests that band intensities are not the consequence of differences in total RNA quantity or quality, but rather reflect kinesin mRNA levels. Thirdly, identical results were obtained with a second set of KLC-3 specific primers. It is concluded that KLC-3 is expressed at higher levels in spermatids than spermatocytes.

ii) KLC-1 and 2 are not expressed in spermatids: previous immunofluorescence experiments in our laboratory with KLC antibodies, which identify multiple KLC isoforms, failed to detect a signal in spermatids. This suggested that KLC-3 was the sole KLC expressed in spermatids. This was pursued by analyzing KLC-1 and 2 expression by RT-PCR as above. Primers were based on published sequences. The authenticity of the bands was established as follows: for KLC-1, the primers amplified a band of equal

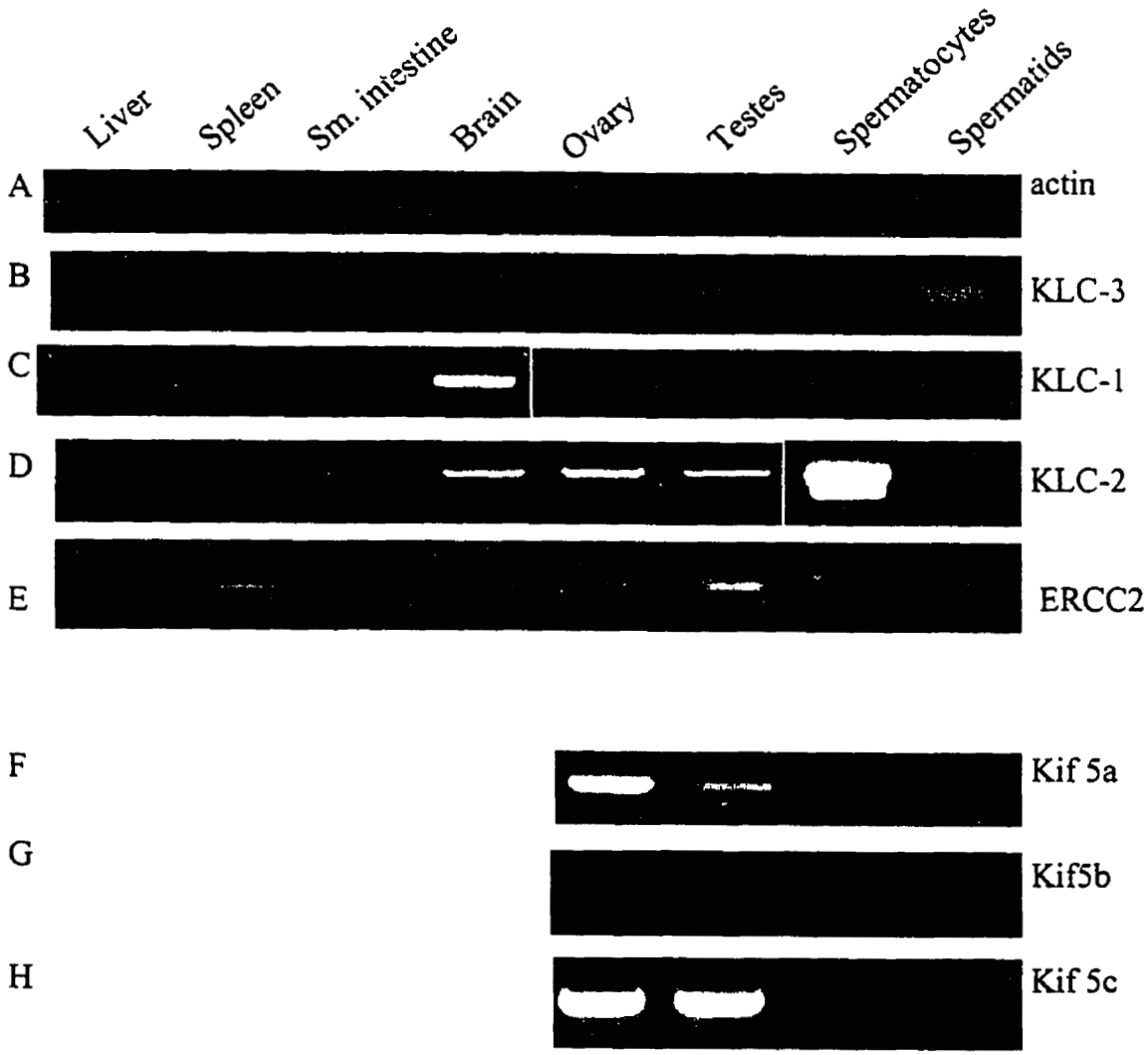
size from a control KLC-1 DNA harbored in a plasmid. Furthermore, in parallel experiments this band was cloned and sequenced and determined to be KLC-1. KLC-2 results were reproduced with an independent set of primers. Results of the RT-PCR are shown if Fig. 10C and D. The results of tissue expression match those obtained by Rahman et al,1998 and confirm our conclusion that PCR band intensities match kinesin mRNA levels. Novel, and of note here, is that both genes are expressed at high levels in spermatocytes, but are undetectable in spermatids. It is concluded that KLC-3 is the only known KLC expressed in spermatids.

iii) Kif 5c is the only KHC expressed in spermatids: in order to determine which KHC could interact with KLC-3 in spermatids, RT-PCR analysis was conducted with primers targeted to one of the three KHC isoforms (Kif5a, 5b or 5c). Of these, only kif5c primers were able to amplify Kif5c DNA, and this band matched that obtained from the various cDNA samples with Kif5c primers. For Kif 5b and c, the PCR product authenticity was verified by restriction analysis. Results for KHC RT-PCR reactions are shown in Fig.10 4E, F and G (Kif5a, b and c respectively). Kif5a and c had previously been reported to be neuronal specific genes, but both were detected in testis. All three genes appear to be expressed in spermatocytes, however only Kif5c was detected in spermatids. Although the Kif5c band was extremely faint in spermatids, direct comparison with KLC-3 expression levels is not possible as different primers were used. It is concluded that only Kif5c can interact with KLC-3 in spermatids, and that Kif5c expression in testis does not closely match that of KLC-3.

Figure 10

RT-PCR analysis of gene expression in various tissues and germ cell fractions: RNA was isolated from various tissues or germ cells, quantified and standardized, used to make cDNA and this amplified with primers specific for the genes indicated. In all cases, band size matched the predicted band size, no-RT controls did not generate bands, and the results were reproduced independently with different primers, or the band identity was verified by restriction analysis. The PCR is assumed to be roughly quantitative based on the rationale described in the text. Note the widespread distribution of KLC-3 in various tissues. KLC-3 expression is highest in spermatids, unlike KLC-1 or 2 which are undetectable here, suggesting KLC-3 may be the sole KLC in spermatids. The second band in this lane is of unknown identity, was not seen with a different set of primers, and is assumed to be a nonspecific product. Note also the absence of Kif5a and 5b, and the relatively low levels of Kif5c expression in spermatids.

10


Liver
Spleen
Sm. intestine
Brain
Ovary
Testes
Spermatocytes
Spermatids
A
actin
B
KLC-3
C
KLC-1
D
KLC-2
E
ERCC2
F
Kif 5a
G
Kif5b
H
Kif 5c

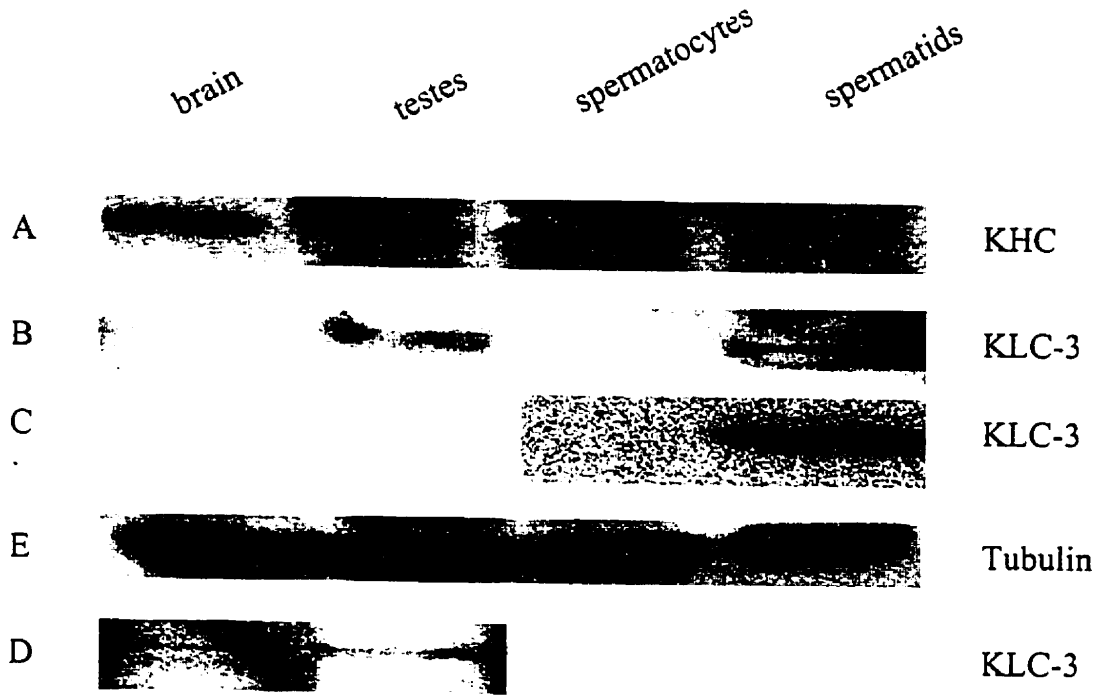
III.c: Western Analysis Corroborates RT-PCR Results

RT -PCR analysis showed that KLC-3 and Kif5c are the only kinesin genes in spermatids, and hinted at differences in expression levels between the two. In order to pursue these ideas, Western analysis of kinesin gene expression was performed. For this purpose, brain and testes tissues and elutriated spermatid and spermatocyte cells were homogenized and lysed in SDS buffer. The protein content was measured by spectrophotometry and commensurate dilutions prepared. Control experiments with secondary antibody dilutions showed that background binding of the antibody to all testes derived samples was equivalent, suggesting approximately equal protein content was achieved. This was verified by conducting Western blots with antitubulin antibodies. As shown in Fig 11E, this antibody generates an equivalent signal for all samples. It is concluded that protein content normalization was sufficiently accurate and suitable for quantitative Western analysis.

i) KLC-3 Westerns corroborate the RT-PCR: KLC-3 polyclonal antibodies were made, and these were used to probe KLC-3 expression by Western. The antibody reliably produced only one specific band in various Westerns. The band is smaller than 62 kDA, and thus cannot represent either KLC-1 or 2, but most likely represents KLC-3 (56kDa). The antibody was therefore used to compare expression levels of KLC-3 in brain, testis, spermatocytes and spermatids. The results are shown if Fig.11B, C and D. At shorter exposures (11B) the pattern of this band matches with the KLC-3 RT-PCR band intensities: the band is too faint to see in brain or spermatocytes, is detectable in the testes, and is strongest in spermatids. Even at longer exposures (11C), the band is not detectable in spermatocytes. 11D shows that at longer exposures, KLC-3

Figure 11

Western analysis of gene expression in brain, testis and germ cell fractions. Samples were standardized for protein content, as indicated by the blot showing constant levels of tubulin in the various samples (11E). The expression of KHC was analyzed by using H1 and H2 antibodies. The blot shown does not discriminate between Kif5b and 5c, as their molecular weights are very similar, but does detect Kif5a. Note the very low levels of expression in spermatids, the band here is barely visible (panel A). The second faint band in the spermatid lane is thought to represent nonspecific background, based on control experiments (panel A). KLC-3 expression was probed using polyclonal antibodies developed in our lab, and detects KLC-3 expression in the testis and spermatids (panel B). At longer exposures (panel D), a band is also detectable in brain, but not is spermatocytes even at long exposures (panel C).


brain
testes
spermatocytes
spermatids
A
KHC
B
KLC-3
C
KLC-3
E
Tubulin
D
KLC-3

is detectable in the brain. It is concluded that the polyclonal is specific for KLC-3, and further that KLC-3 is expressed at high levels in spermatids.

ii) KHC Westerns corroborate the RT-PCR, with some caveats: KHC isoform expression was probed with commercially available H1 and H2 monoclonal antibodies. These antibodies are reported to recognize kif5b, and were able to recognize *in vitro* translated kif5c. This is consistent with reports that the antibodies detect Kif5c (Goldstein lab, pers com). Western blots with these antibodies generated a single band in total brain lysates, and a corresponding (and weaker) band in total testis lysates and spermatocytes, and a very weak band with spermatid extracts (Fig 11A). Longer exposures (from an identical but different blot) demonstrate the presence of this band in spermatids more clearly (11B). Since Kif5c and b differ in length by only 7 amino acids (956 vs. 963), which translates to less than 1 kDa, it is likely that the single band generated in the western blots represents a sum of Kif5b and 5c. Kif5a or other potential novel kinesins were therefore not detected by these antibodies. Kif5b and/or 5c are thus expressed in total testis, in spermatocytes and at lower levels in spermatids. In conjunction with the RT-PCR results, we conclude that the band detected in the spermatid fraction is derived from Kif5c, and this gene may interact with KLC-3 in spermatids. The expression profile does differ somewhat from the RT-PCR results: note that spermatocyte expression may be equal to total testes. This may represent either an experimental artifact or post-transcriptional control mechanisms. Nonetheless, Kif5c expression levels in spermatids appear low, and may not match KLC-3 expression levels. This raises the possibility that the excess KLC-3 either interacts with a novel KHC or does not interact with any KHC in spermatids.

III.d: KLC-3 is the sole kinesin expressed in the sperm tail

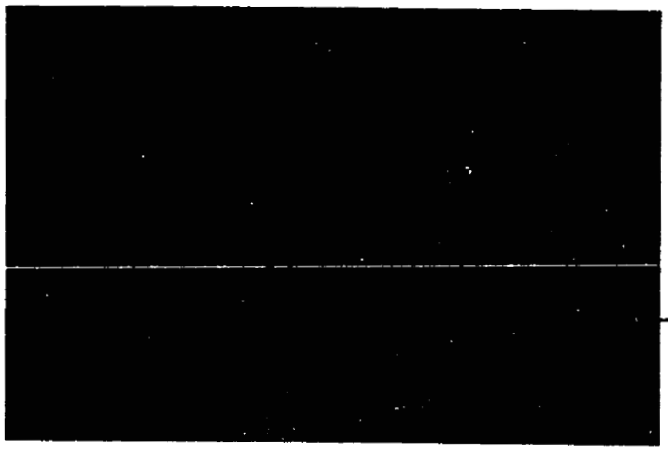
i) KLC-3 monoclonal localizes to sperm tails via Immunofluorescence:

immunoprecipitations and westerns, conducted previously in our lab, demonstrated that the 11g6b2 monoclonal antibody is specific for KLC-3 (van der Hoorn, per coms). Thus this antibody was used in immunofluorescence experiments of testis cross-sections. This was done both to verify previous observations, and to compare with Kif5a/c immunofluorescence. Testis cryosections were also probed with, ODF-2 polyclonal antiserum, which specifically stains sperm tails (Shao et al., 1997) and the DNA dye DAPI. A representative low magnification series is shown in Fig. 12. Several ST cross-sections are evident in the picture, as can be deduced from the DAPI stain (12A). As is readily evident, the KLC-3 and ODF-2 patterns are essentially the same, indicating that the KLC-3 monoclonal is specifically staining sperm tails (compare 12B and C). Higher magnification pictures show fluorescence of both short (elongating) and long sperm tails (12E and G). It is concluded that KLC-3 protein localizes primarily in sperm tails, in agreement with previous results. However, we cannot rule out low levels of expression in other cell types or subcellular compartments.

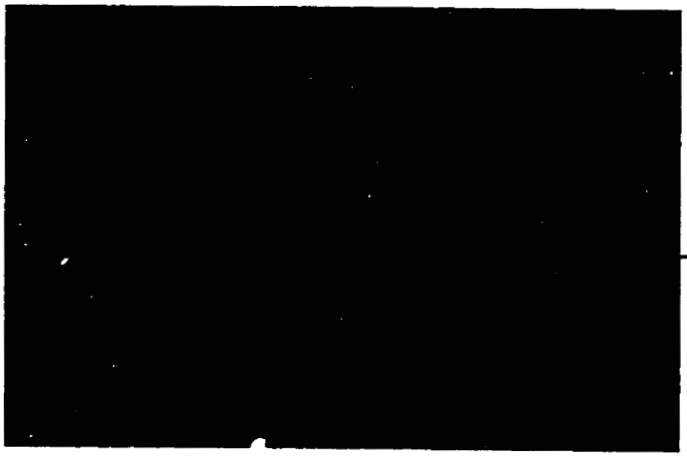
Figure 12

The expression of KLC-3 in seminiferous tubules (ST) was examined with a monoclonal specific for KLC-3(van der Hoorn, per coms) in ST cryosections by immunofluoresence. DNA and ODF-2 expression was used for comparison A) DNA stain (DAPI) of a cross-section of the seminiferous tubule showing parts of three tubules. B) the same view, showing ODF-2 expression, a major structural component of the outer dense fibers sperm tails. C) Expression of KLC-3 is also specific for sperm tails. The staining of ST periphery is thought to be due to nonspecific binding of the secondary antibody, based on control experiments using only secondary antibody. D-G) Higher magnifications of the sperm tail staining of KLC-3, with DAPI staining provided for contrast.

12A



B



C

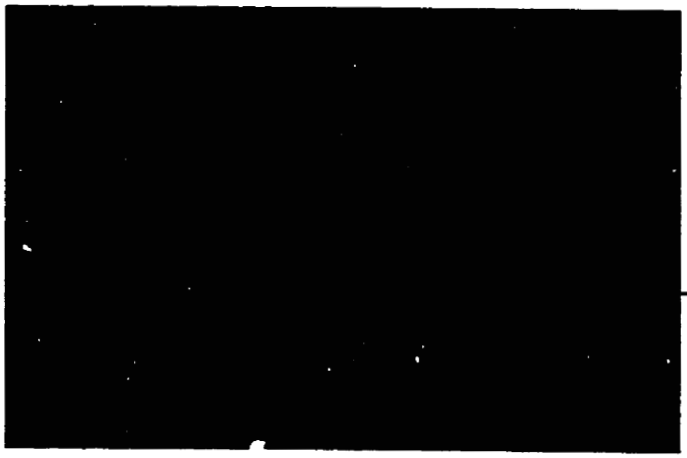


Fig. 12

D

E

F

G

iii) KHC antibodies do not generate a signal in sperm tails: KHC antibodies have been used previously to show that expression in the testes is confined to the manchette of spermatids and the trans-Golgi of Sertoli cells(Johnson et al., 1996b; Hall et al., 1992). To verify these observations and rule out the possibility of KHC expression in sperm tails, immunofluorescence experiments were conducted with H1 and H2 antibodies. As demonstrated above, these antibodies likely recognize Kif5b and 5c in the testes. Experiments were done as for KLC-3, and a representative series is shown if Fig. 14. As is clearly evident from the ODF-2 and DAPI staining (Fig. 14A and B) several STs containing sperm tails are represented. However, no corresponding tail staining can be seen in with the KHC antibody (14C). Instead, the antibodies stain punctate structures, which are confined largely to the perimeter of the ST (see also Fig. 14E). There was some variability in this staining, sometimes more diffuse (E) and sometimes more punctate (C) in appearance. Fig. 14E shows a pattern reminiscent of vesicle staining. Identical results were obtained with KHC specific antibodies received from Dr. B. Schnapp. It is concluded that Kif5b and 5c do not co localize with KLC-3 in sperm tails.

Figure 13

The expression profile of KHC genes in the ST was analyzed by immunofluorescence using three separate KHC specific antibodies. All three gave the same results. A) DAPI stain B) ODF-2 staining (sperm tails) of the same section C) KHC staining of the same section. Based on western and RT-PCR analysis of KHC gene expression, the signal is thought to represent Kif5b and/or 5c. Note the punctate pattern of KHC expression, and the absence of staining in sperm tails. The source of this staining is unknown. D and E) higher magnifications of the punctate staining pattern.

Figure 13 A-E KHC IIF

13

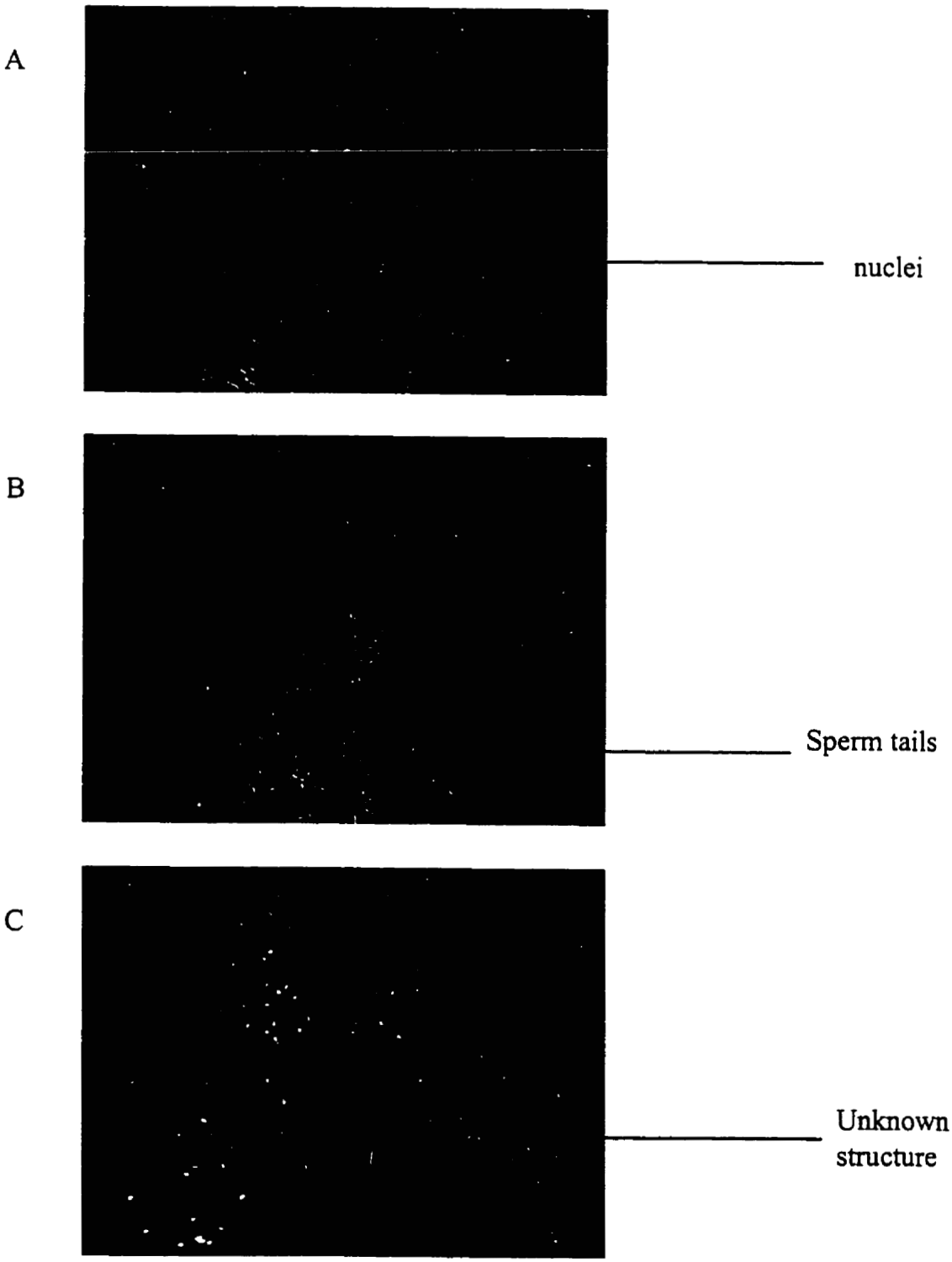
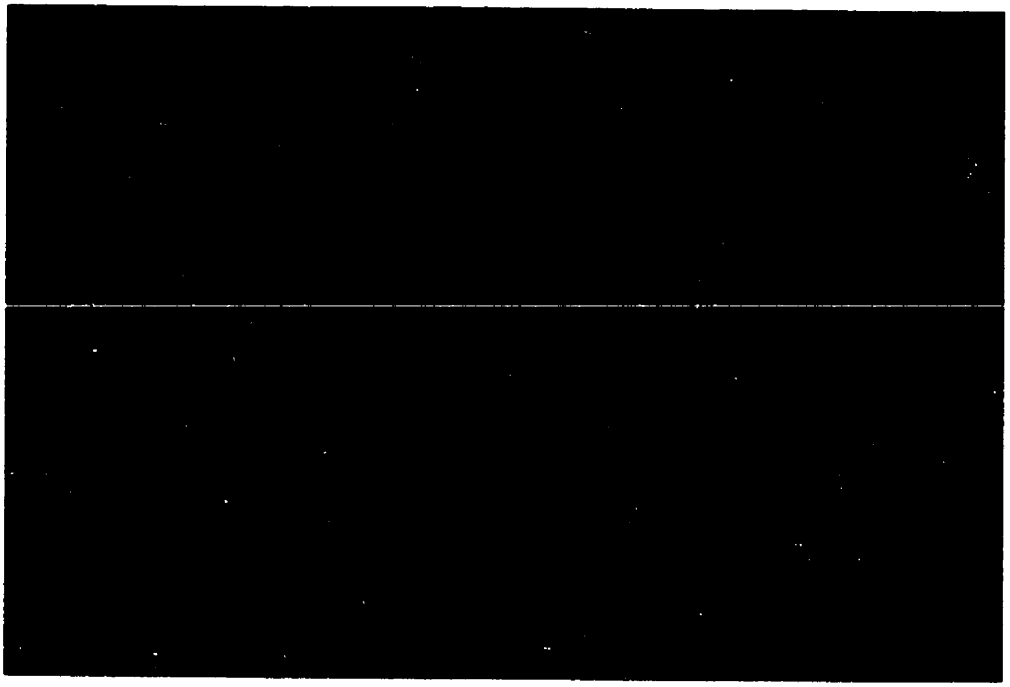
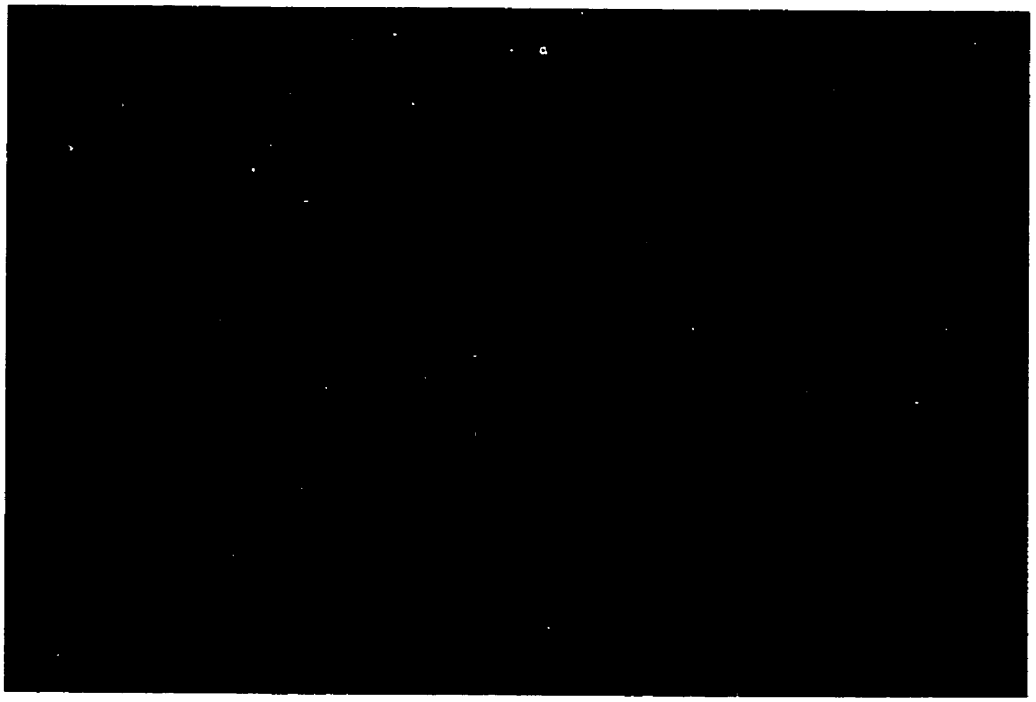


Fig.13D



E



III.e: KLC-3 can interact with Outer Dense Fiber Proteins

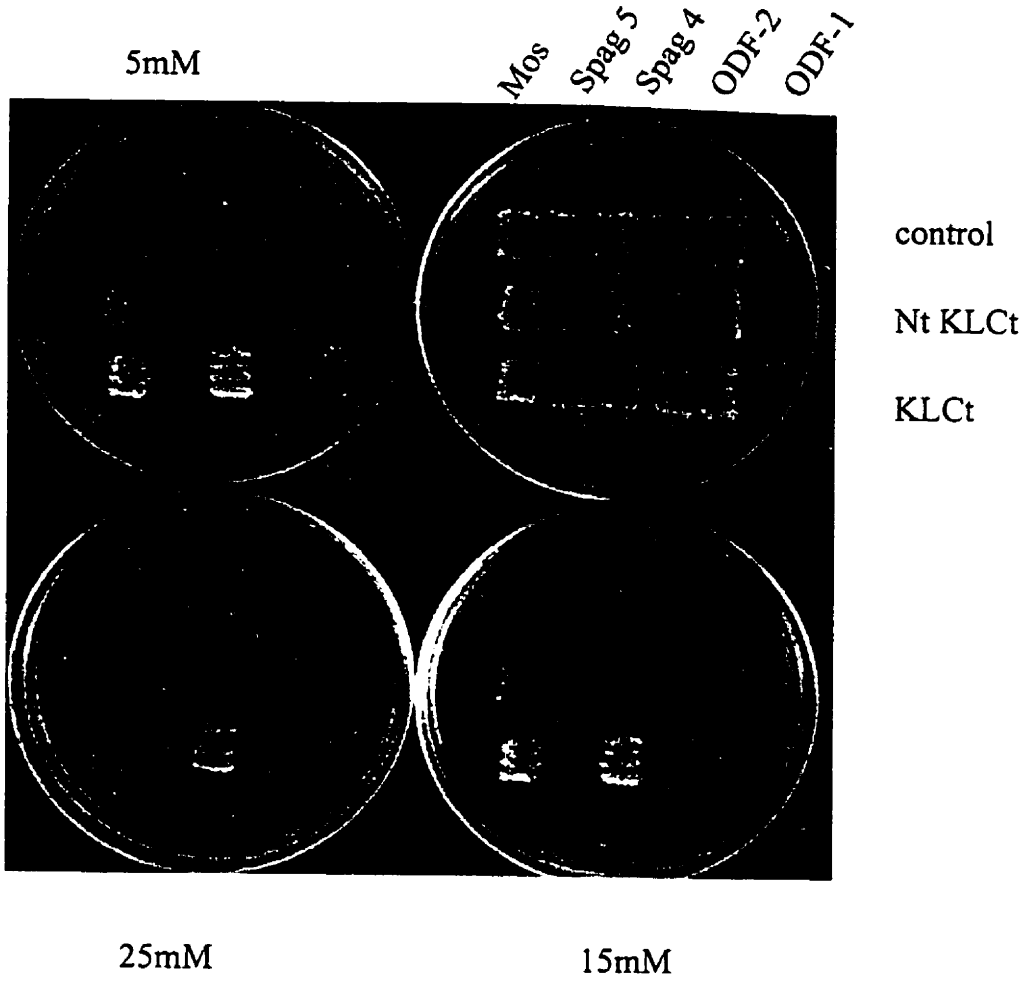
i) KLC-3 interacts with ODF-1 and SPAG4 in yeast two hybrid: The lack of KLC-3 and Kif5c co-localization in spermatids suggest either an unusual function for KLC-3 or interaction with a novel KHC. The previous localization of KLC-3 to ODF by our lab(van der Hoorn, per coms), and the lack of MTs in this structure (van der Hoorn, per coms) support the former possibility. In order to further investigate the localization of KLC-3 to ODFs and suggest possible functions, the interaction of KLC-3 with various ODF proteins in the yeast two-hybrid system was tested. This system has been used to characterize both ODF protein interactions, and the interaction of Mos with KLC-3 ((Shao and van der Hoorn, 1996; Shao et al., 1997; Shao et al., 1999; van der Hoorn, per coms). KLC-3, N-terminal KLC-3 or empty vector was co-transformed with either ODF-1, ODF-2, SPAG4, SPAG5 or Mos (as a positive control for KLC-3) or empty vector (as a negative control), in the yeast strain MaV203. Clones were picked and streaked onto a master plate, which was used to test activation of the His and Lac Z promoters. Ability to activate His promoter was done by replica plating onto successively higher concentration of the His enzyme inhibitor 3-AT. Interaction of the proteins was concluded if growth was observed for the experimental but not the control colonies. The results are summarized in Fig. 15: Mos, SPAG4 and ODF-1 where found to interact with KLC-3 via this assay.

ii) KLC-3 interaction with ODF-1may be mediated by the ODF-1 Leucine Zipper: Leucine zippers have been implicated in ODF protein interactions, and sequence analysis of KLC-3 revealed a Leucine Zipper motif within the HR region. It is hypothesized that

Figure 14

Yeast two hybrid analysis of KLC-3 interaction with some cloned outer dense fiber proteins, ODF-1, ODF-2, Spag4 and Spag5. c-Mos is used as a positive control. An N-terminal fragment of KLC-3, which incorporates the HR region and a stretch of the TPR was also used (refer to fig. 17A) to roughly map the interacting domains. Empty vector was used as a control. Yeast strain MaV203 was transformed with the genes indicated, and activation of the His promoter analyzed by plating on -His plates with successively higher concentrations of 3-AT. Interaction is scored if the plasmid control shows no growth but the KLC-3 or Nt-KLC-3 clones do grow. In this assay, c-Mos, ODF-1 and Spag4 were found to interact with KLC-3.

Fig. 14



this motif of KLC-3 might mediate interaction with ODF protein Leucine Zippers. In order to test this hypothesis, assays on interaction of KLC-3 and N-terminal KLC-3 with various ODF-1 constructs (Fig. 16A(Shao and van der Hoorn, 1996)) in yeast two hybrid were conducted. The results are shown in Fig. 16B. ODF-1 deletions of C-terminal ODF1 sequences did not affect interaction with N-terminal or FL KLC-3, while deletion of N-terminal ODF1 sequences abolished the interaction. A mutant of ODF-1 lacking leucine zipper sequences did not interact with KLC-3. It is concluded that the leucine zipper of ODF-1 likely mediates interaction with (N-terminal) KLC-3. Subsequently, KLC-3 deletion constructs were generated (Fig. 17A), and assayed these for interaction with N-terminal ODF-1. Only full length and N-terminal KLC-3 supported interaction with ODF-1, whereas neither TPR sequences nor HR deletion mutants did (Fig. 17B). Collectively, this suggests that Leucine Zippers mediate the interaction between KLC-3 and ODF-1. However, based on the available evidence it is not possible to rule out the entire KLC-3 HR motif in the interaction.

iii) KLC-3 interaction with SPAG4 is likely mediated by the KLC-3 Leucine Zipper: the same KLC-3 constructs used to assay interaction with ODF-1 were used in analogous experiments with SPAG4. The results obtained (Fig 17C) are comparable to those obtained for ODF-1, and implicate either the leucine zipper or the entire heptad repeat of KLC-3 in the interaction: only full length and N-terminal KLC-3 supported interaction with SPAG4, whereas neither TPR sequences nor HR deletion mutants did. As for ODF-1, the leucine zipper hypothesis is favoured, as these have been shown previously to mediate interactions for these proteins.

Figure 15

A)ODF-1 constructs used to map the interaction with KLC-3. The constructs were generated previously in our lab(Shao and van der Hoorn, 1996). The two major motifs in ODF-1 are the N-terminal leucine zipper and the C-terminal CGP repeats. The constructs are targeted to include either of these two motifs. B) yeast two hybrid analysis of KLC-3 interaction with the ODF-1 deletion constructs. The assay tests activation of the His promoter, scored by growth on –His plates at successively higher 3-AT concentrations. N-terminal KLC-3 (Fig. 17A) was used to roughly map the KLC-3 motifs. Empty plasmid was used as a control. Growth, and hence interaction, is evident for KLC-3 and to a lesser extent N-terminal KLC-3 with both of the N-terminal ODF-1 constructs, but not an N-terminal construct which excludes the leucine zipper or the C-terminal constructs. Hence, KLC-3 likely interacts with the ODF-1 leucine zipper. B)figure 16 (odf deletions vs KLC-3

Fig.15A

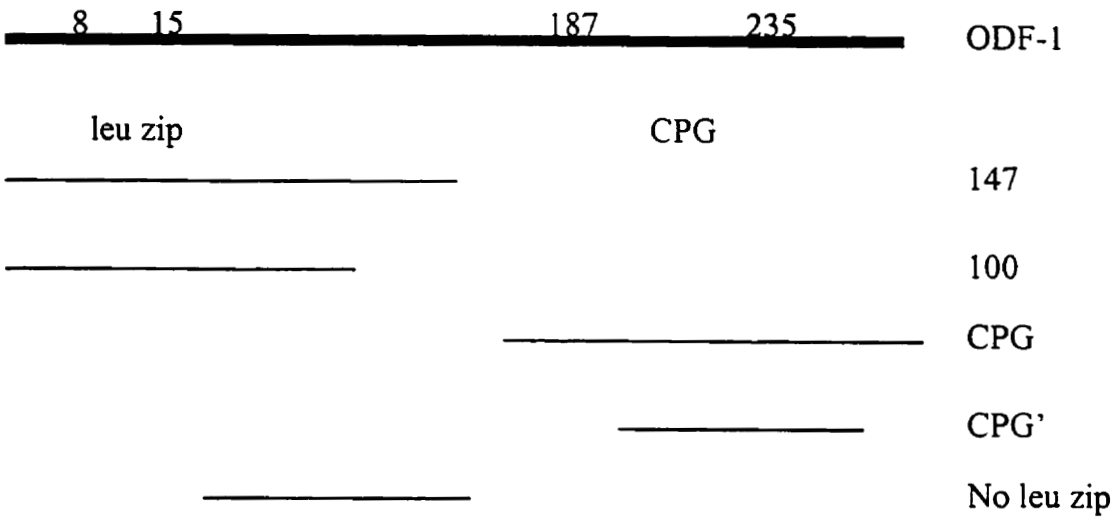


Fig. 15B

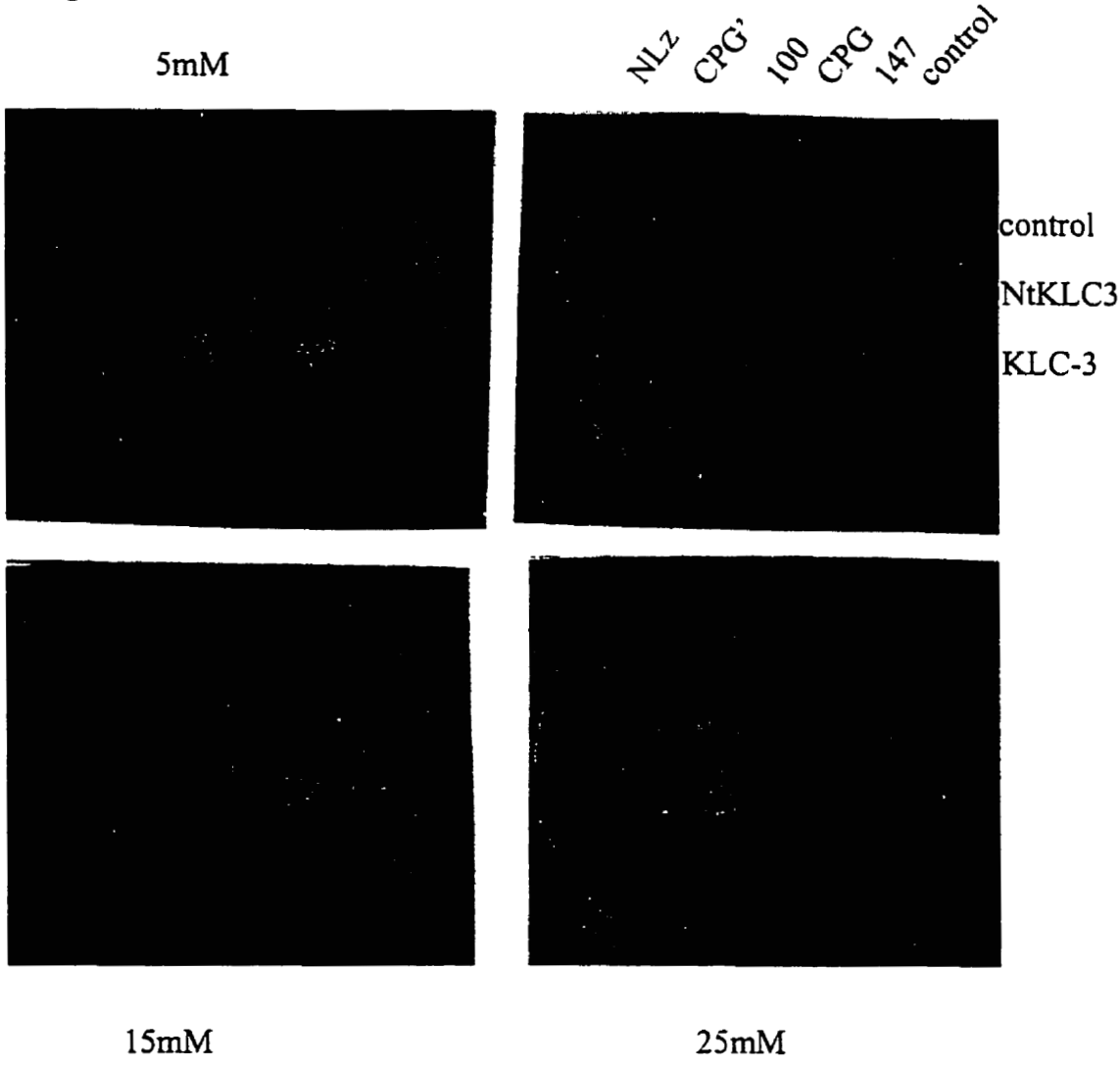


Figure 16

A) KLC-3 constructs used to map the domains mediating interaction between KLC-3 with ODF-1 (fig.17B), Spag 4 (fig.17C), and c-Mos (fig.21). The deletions were made to target the three main motifs of KLCs: the HR domain, which is the only domain present in "1-726" and lacking in "delta HR", the TPR domain, the only domain in "TPR", and partially absent in "1-833", and the C-terminal domain, which is absent in both 1-726 and N-terminal constructs. No insert plasmid is used as a negative control. B) Yeast two hybrid analysis of KLC-3 construct interaction with N-terminal ODF-1 ("100" construct) or plasmid control. Activation of the His promoter was assayed by growth on –His/3-AT plates. Only KLC-3 constructs incorporating the HR domain (full-length and 1-726, lanes 1 and3) support interaction with ODF-1. Deletion of HR abolishes this interaction (lane 2). Thus the HR domain, or the leucine zipper within it, likely mediate interaction with ODF-1. C) The analogous experiment with Spag 4. Results are the same as those obtained with ODF-1, although the interaction is considerably weaker.

Fig. 16A

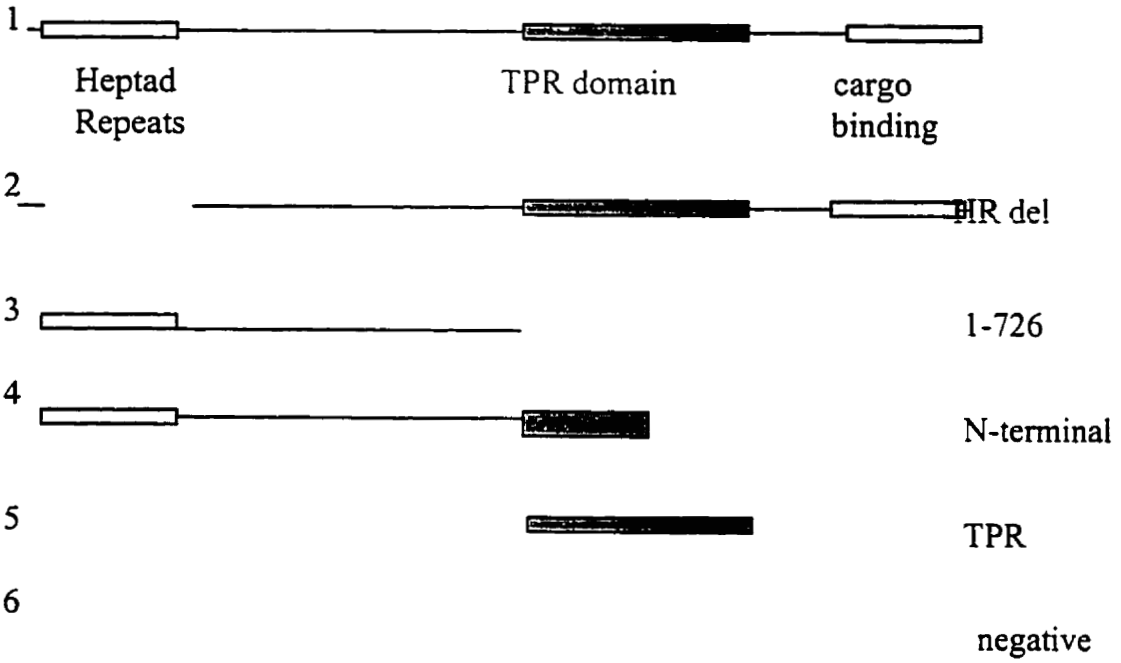


Fig. 16B

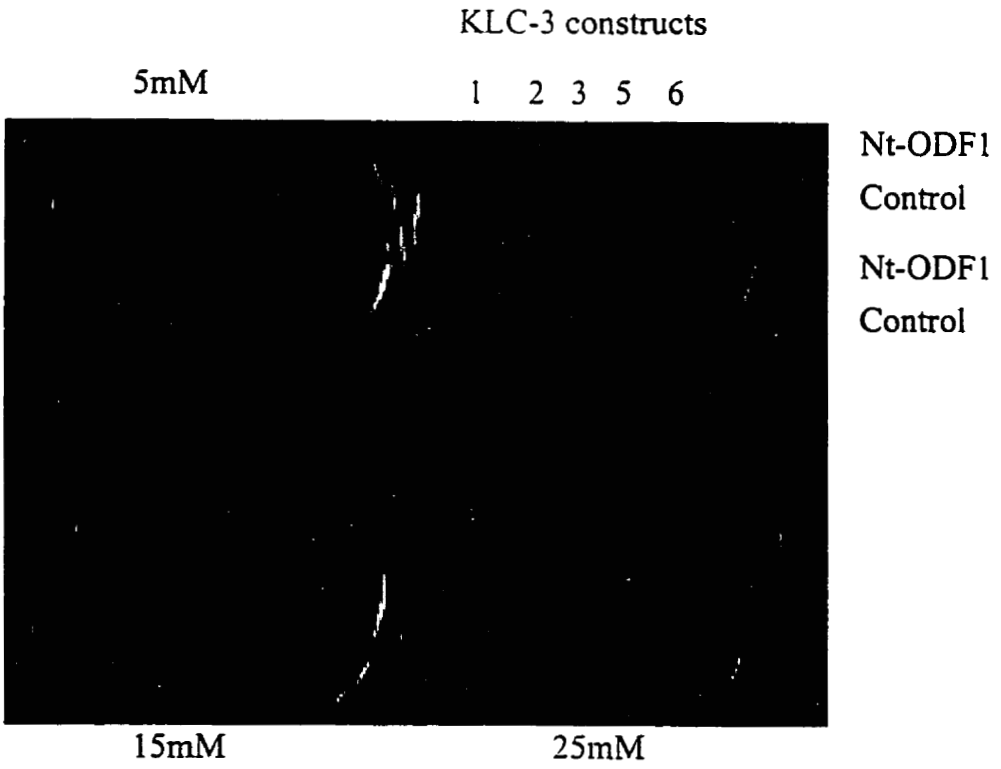
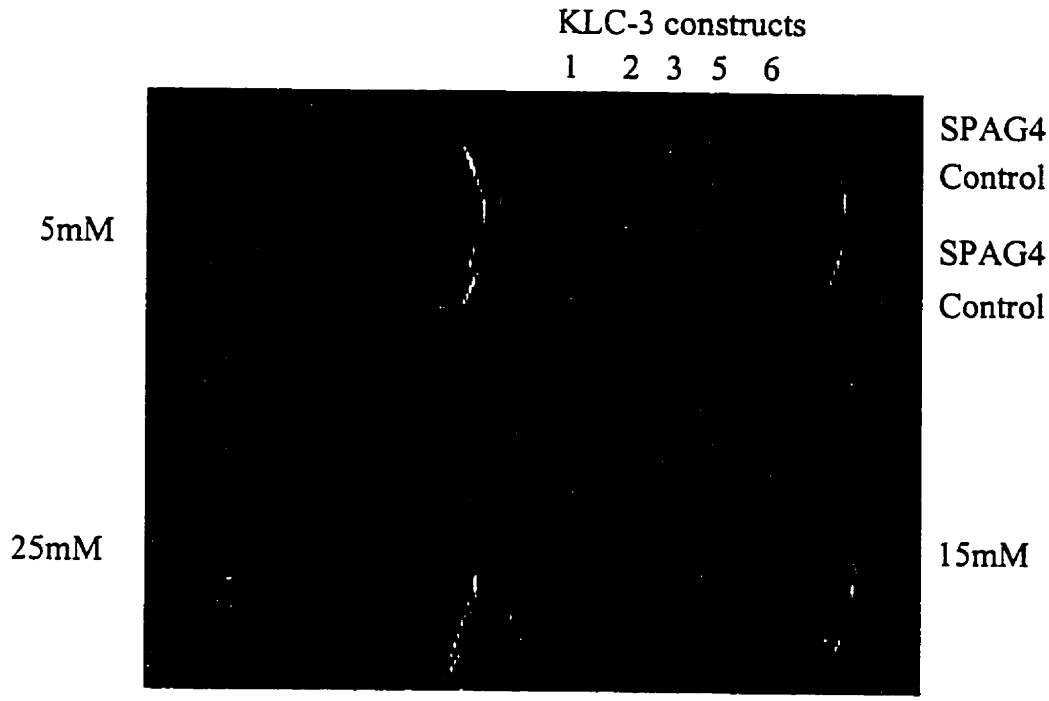


Fig. 16.C


KLC-3 constructs
1 2 3 5 6
SPAG4
Control
SPAG4
Control
5mM
25mM
15mM

iv) Immunoprecipitations of KLC-3 with SPAG4 and ODF-1 are unsuccessful: The yeast two-hybrid system can generate both false positives and negatives. To confirm the interaction of KLC-3 with SPAG4 and ODF-1, the interaction was tested via immunoprecipitations. The experiments were conducted as described for KLC-3 and Kif5c, except a different buffer was used which has been used previously to test leucine zipper interactions in our lab (Shao et al., 1999). As shown in Fig. 18A, SPAG4 and ODF-1 both precipitated in the absence of KLC-3. This suggested problems with either background binding to beads, or problems unique to the insoluble nature of ODF proteins. When the experiment was repeated using the same conditions used for the Kif5c immunoprecipitation, SPAG4 did not co-immunoprecipitate with KLC-3 (fig. 18B). This suggests that SPAG4 and KLC-3 do not interact under these conditions, and questions the results obtained in the yeast two-hybrid assay. It is concluded that alternative protein interaction experiments are needed to ascertain whether KLC-3 interacts with ODF proteins. The hypothesis that it does interact with these structures is favoured, based on its immunolocalisation to these structures.

IV. c-MOS INTERACTION WITH KLCs

IV.a: c-Mos interaction with other KLC isoforms

i) Mos interacts with some KLCs in yeast two hybrid: Results obtained in our lab demonstrated that KLC-3 and c-Mos interact. In order to determine whether other KLC genes are capable of binding to c-Mos, the interaction of c-Mos with mKLC-1, mKLC-2, rKLC-3 and N-terminal (Nt) fragments of rKLC-1 and mKLC-1 and rKLC-3 was tested in yeast two hybrid experiments. The yeast strain HF7c was used to test activation of the

Figure 17

Testing the interaction between KLC-3 and c-Mos, ODF-1 and Spag4 by Co-immunoprecipitation. The proteins were transcribed, translated and radiolabelled in *vitro*, and then immunoprecipitated (Ip) with antibodies targeted to KLC-3, and analyzed by SDS-PAGE and autoradiography. A) ODF-1, Spag4, c-Mos and ODF-2 were either individually translated (- lanes), or co-translated with KLC-3 (+ lanes) and then immunoprecipitated. All proteins (except ODF-2, which did not translate) were found to precipitate both in the presence and the absence of KLC-3. Thus, the interaction could not be determined by this method. B) Spag4 interaction with KLC-3 was investigated in a different Ip buffer. This buffer had worked previously for KLC-3/Kif5c interaction (fig. 8). The experiment was done simultaneously with that in fig. 8A, in the same manner: KLC-3 and Spag4 were translated either individually (last two lanes) or together at different ratios (middle lanes), and then Ip'ed with antibodies targeted to KLC-3. Spag 4 was not found to Ip with KLC-3 in this assay (middle lanes). The TNT reactions were run out beside the Ip's (first two lanes) to demonstrate the presence of Spag 4 in the reaction.

Fig.17A

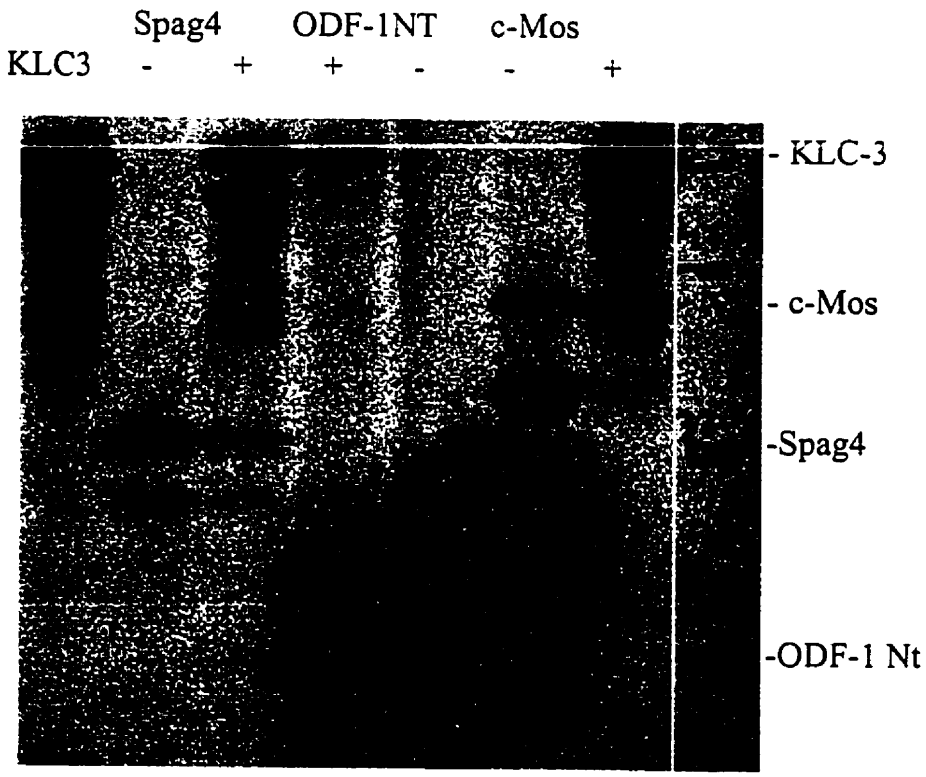
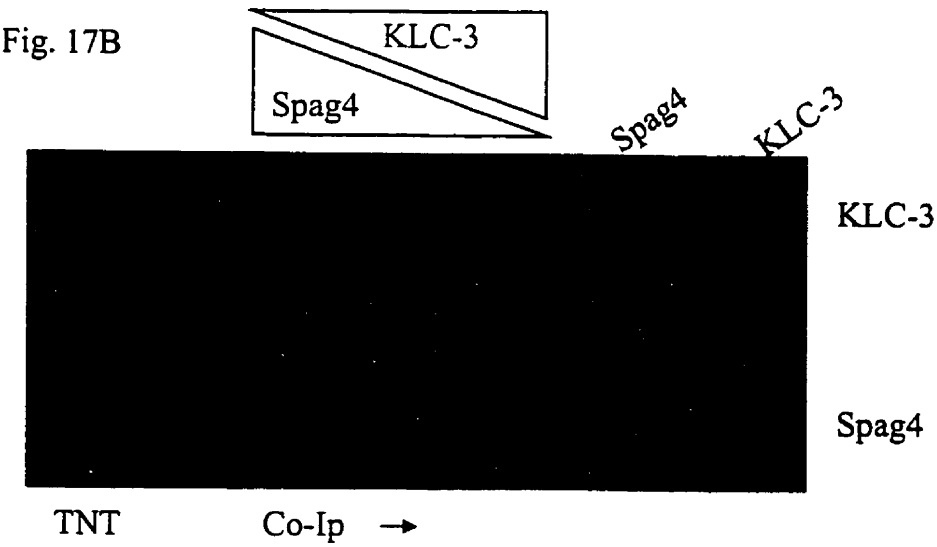


Fig. 17B



His promoter, and SFY526 to test activation of the LacZ promoter. The results of this experiment were ambiguous and difficult to interpret (Fig. 19A and B). rKLC-3 and N-terminal rKLC-3 activated both His and Lac Z promoters, as did KLC-2, although very weakly. N-terminal rKLC-1 also activated both promoters, whereas N-terminal mKLC-1 activated only the His promoter. Full-length mKLC-1 did not activate either promoter. This was unexpected as the sequence conservation between KLC-3 and rKLC-1 (48%) is much lower than that of rKLC-1 and mKLC-1 (95%). It is therefore unlikely that KLC-3 and rKLC-1 have conserved Mos binding sequences, which are not conserved in KLC-1. Thus it is likely that Mos interacts either with both KLC-1 homologues or neither, and it is concluded that one of the two results is an artifact. Furthermore, the interaction with KLC-2 was so weak as to be barely detectable, and may not represent a biologically relevant interaction. In an attempt to clear up the ambiguous results obtained in the yeast two hybrid, I conducted immunoprecipitation experiments as described for KLC-3 and Kif5c. The results of this experiment are also ambiguous, as c-Mos was observed to immunoprecipitate in the absence of co-translated KLC. The indefinite results bar any conclusions.

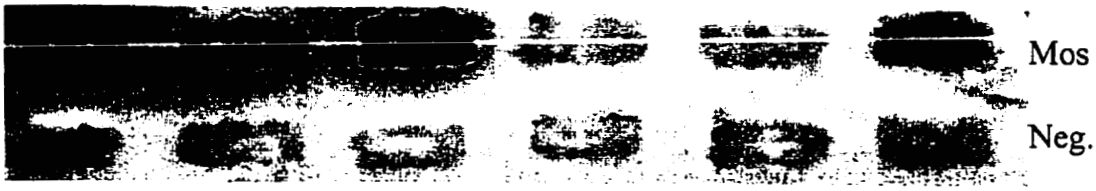
IV.b: KLC-3 interacts with the extracatalytic Domain of c-Mos via its HR region

i) KLC-3 does not interact with the c-Mos kinase domain in yeast two hybrid: the interaction of c-Mos with KLC-3 suggests two possibilities: either KLC-3 is a substrate for c-Mos, perhaps as a mechanism for kinesin regulation, or alternatively KLC-3 could act as a MT tether for c-Mos. The fact that kinase dead c-Mos is capable of mediating

Figure 18

The interaction between c-Mos and various KLC family members was investigated by yeast two hybrid assays. Full-length mKLC-1 and 2 were a kind gift of the Goldstein lab. N-terminal mKLC-1 and rKLC-1 were both cloned by RT-PCR. A) activation of the lacZ promoter in yeast strain SFY 526, and B) activation of the –His promoter in HF7c. For both assays, empty vector was used as a negative control. The results show that KLC-3, Nt KLC-3 and Nt rKLC-1 interact in both systems. MKLC-2 also interacts in both systems, but very weakly. The results for mKLC-1 were inconsistant, demonstrating interaction only for Nt mKLC-1 in the His promoter (but not Lac Z) assay. This is surprising in light of the interaction of c-Mos with the very similar rat homologue (rKLC-1, N terminal fragment). Thus, conclusions are not possible.

Fig. 18A



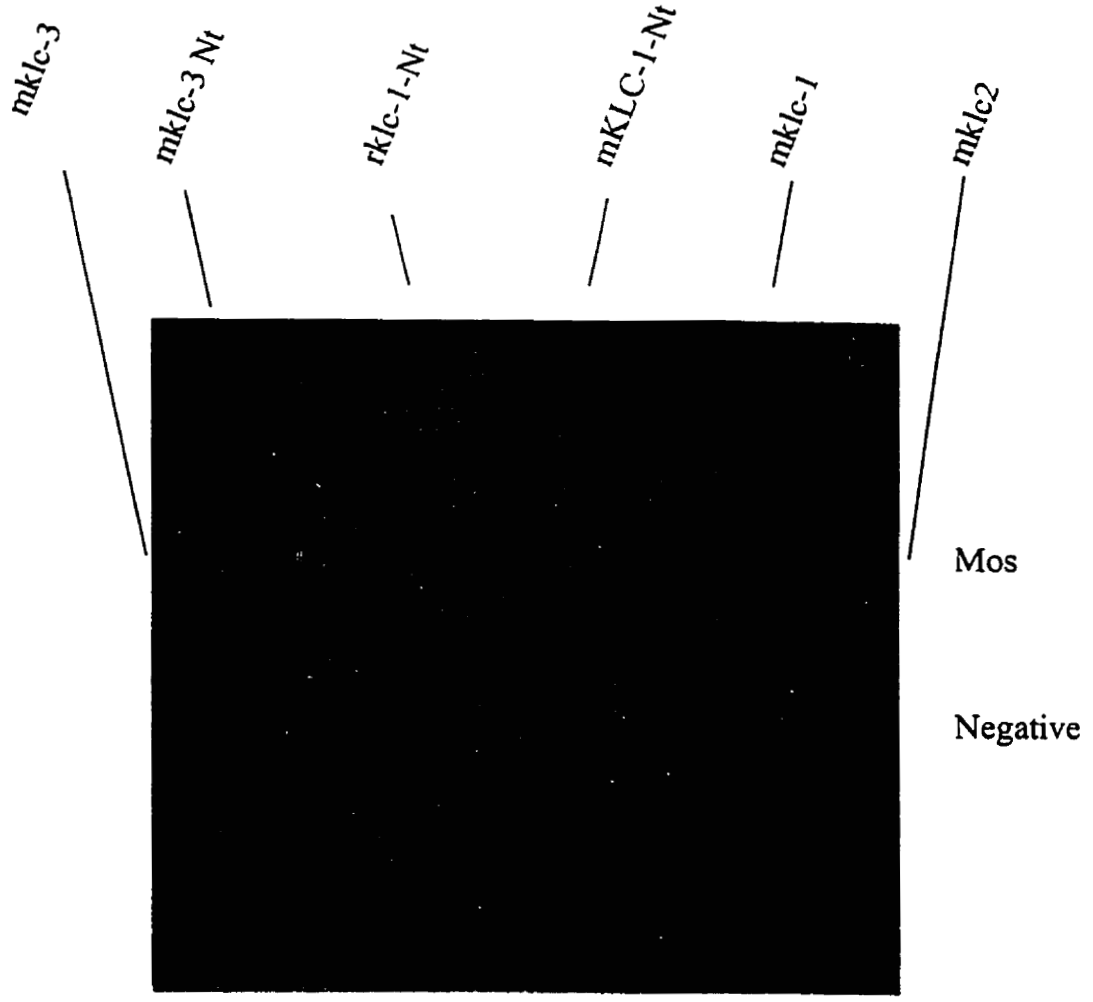


Fig. 18B

Figure 19

The interaction between KLC-3 and c-Mos was mapped by using c-Mos deletion constructs in yeast two hybrid. A) c-Mos motifs and the constructs used to map the interaction. The C-terminal deletions were made by (van der Hoorn, per coms) by using restriction sites, the N-terminal deletions were made by long PCR. B) Results of yeast two hybrid analysis of His promoter activation in yeast strain MaV203. Deletions of the N-terminal 230 or the C-terminal kinase domain (lanes 2 and 5) did not affect the interaction with c-Mos. C) Results of LacZ promoter activation in yeast strain SFY 526: as in the previous assay, only full length, delta 230 and 1-885 constructs supported interaction with c-Mos. Further deletions from either end abolished interaction with KLC-3. Thus, a region of c-Mos within nucleotides 230-885 mediates the interaction with KLC-3.

Fig 19.A

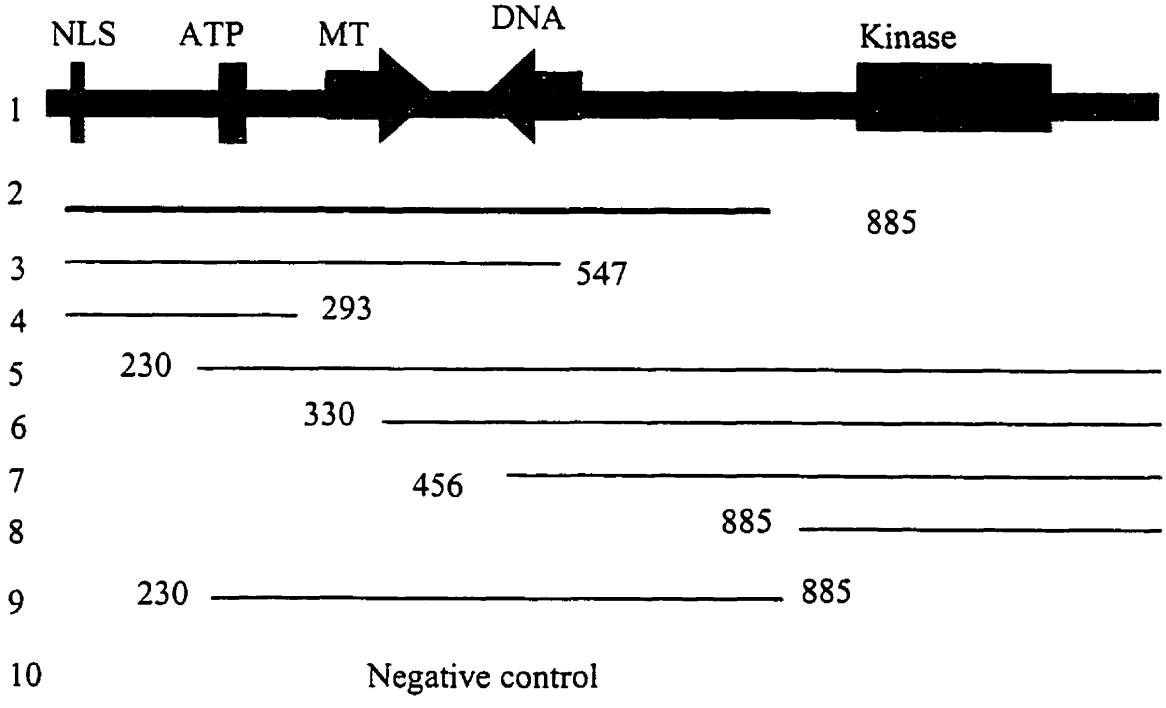

NLS
ATP
MT
DNA
Kinase
1
2
885
3
547
4
293
5
230
6
330
7
456
8
885
9
230
885
10
Negative control

Fig. 19.B

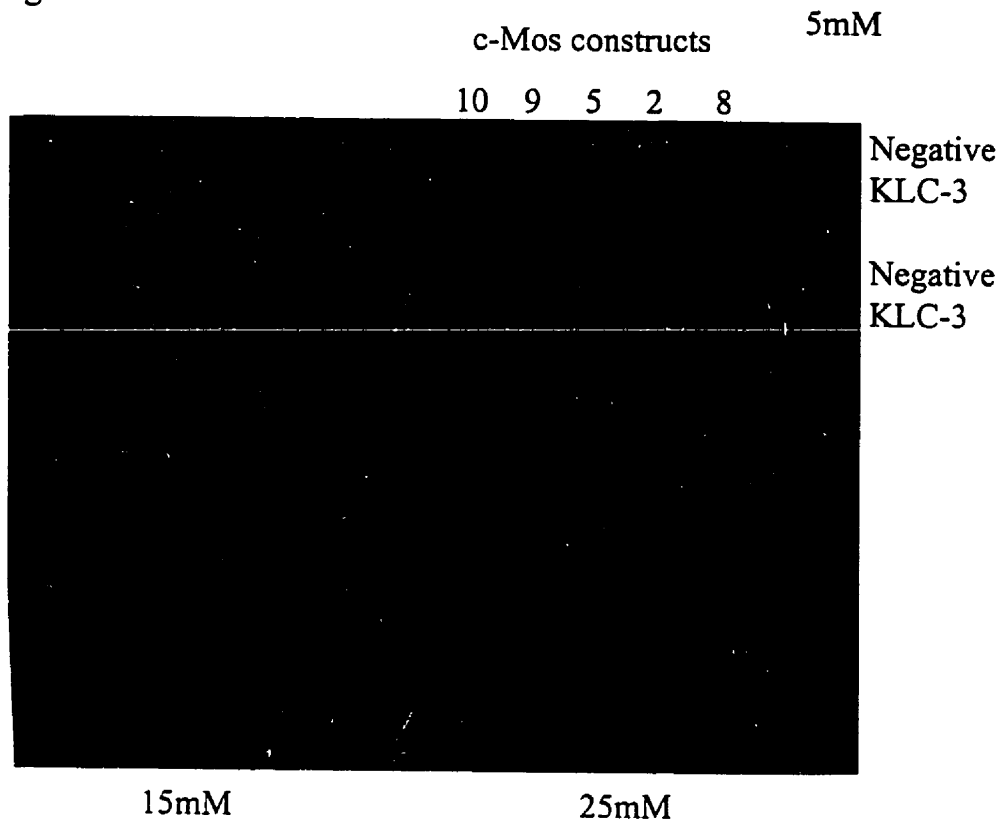
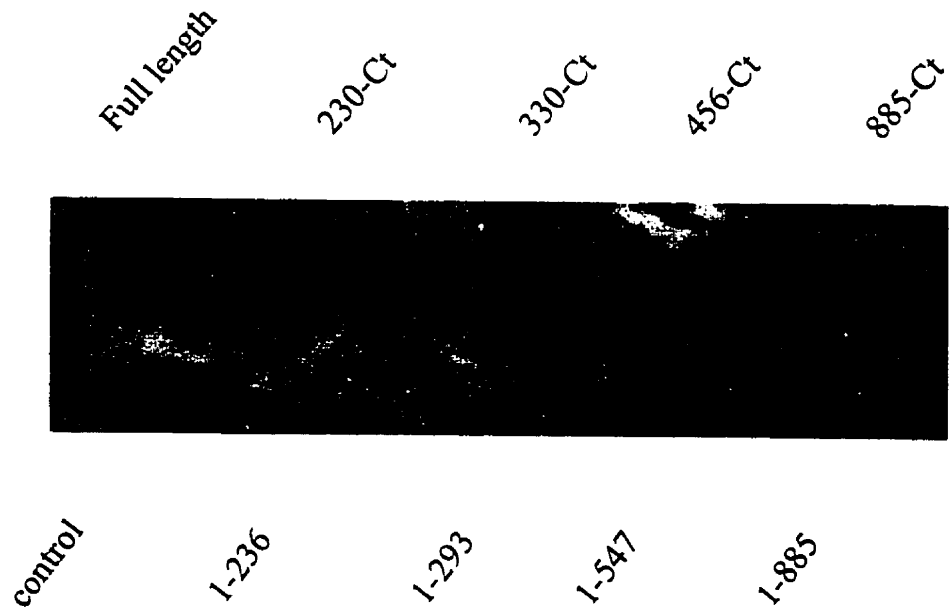


Fig. 19.C



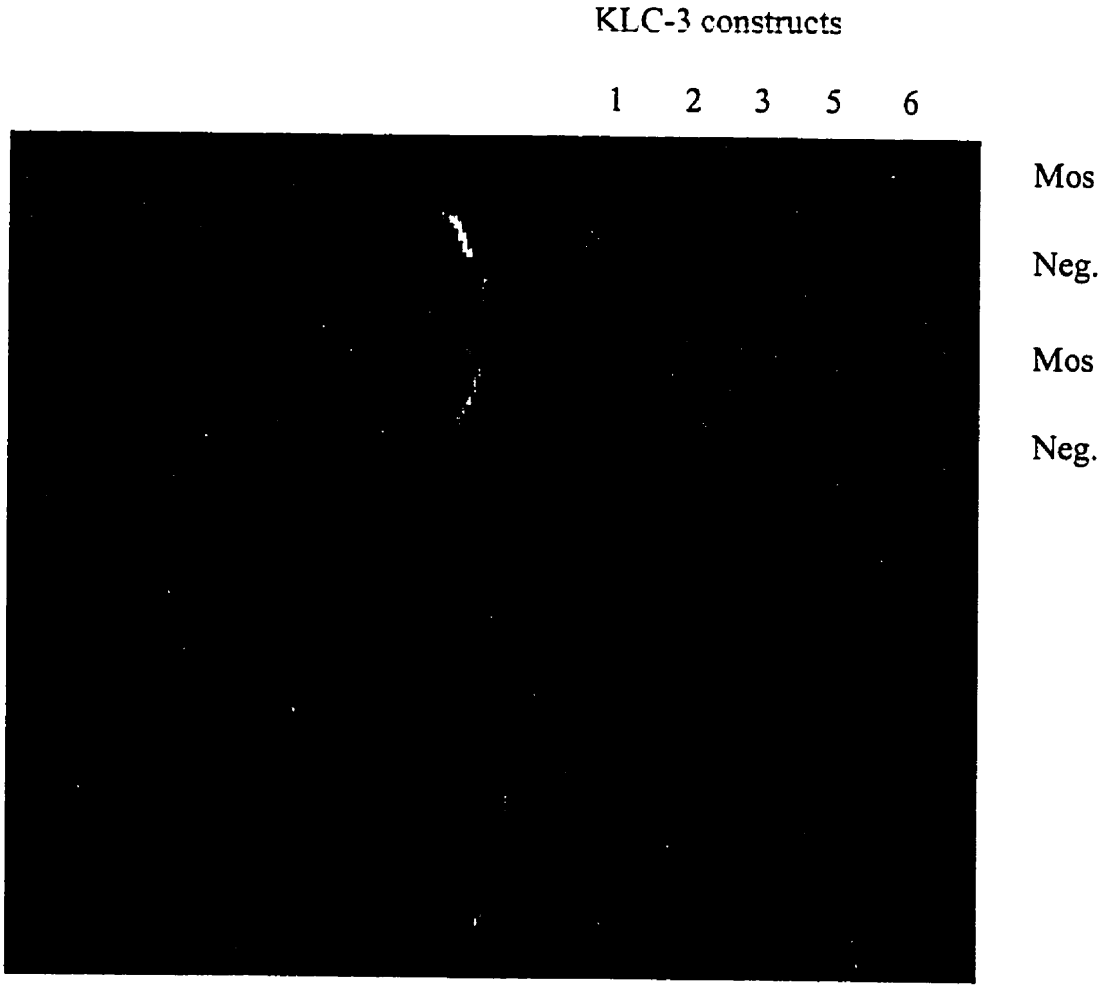
interaction with KLC-3 suggested the latter possibility. In order to substantiate this conclusion, deletion mutants of c-Mos were generated (Fig. 20A). These were used in yeast two hybrid assays as described for ODF-1/KLC-3. The results of His activation are shown in Fig. 20B, and demonstrate that c-Mos lacking the kinase domain (1-885) can interact with KLC-3. The C-terminal half of c-Mos (kinase domain) did not interact with KLC-3. It is concluded that KLC-3 does not interact with the c-Mos kinase domain, and hypothesize that KLC-3 functions as a MT tether for c-Mos. Deletion of the N-terminal 230 nucleotides had no effect on the interaction, whereas a further deletion of 100 nucleotides abolished the interaction. It is concluded that the KLC-3 interacting domain resides between nucleotides 230 and 885 of c-Mos.

ii) KLC-3 interacts with c-Mos via its HR region in yeast two hybrid: in order to determine which domains of KLC-3 mediate interaction with c-Mos, KLC-3 deletion constructs were assayed for c-Mos interaction as described for ODF-1. The results obtained (Fig. 21) were also very similar to those obtained for ODF-1 and c-Mos. A construct spanning amino acids 1-726 (including the HR but excluding the TPR) supported interaction with c-Mos, whereas the HR deletion mutant did not. Constructs spanning only the TPR domain did not interact with c-Mos. It is concluded that the HR region of KLC-3 mediates interaction with c-Mos. In this case, it is not possible to conclude that the leucine zipper was involved: for ODF-1 it was demonstrated that its' leucine zipper mediated the interaction with KLC-3, and for both SPAG4 and ODF-1 there is precedent that leucine zippers engage in interactions with other leucine zippers. However, c-Mos has no leucine zipper. Hence, it is hypothesized that the HR region, rather than the leucine zipper mediates the interaction with c-Mos.

Figure 20

The interaction of KLC-3 deletion constructs (fig. 17A) with c-Mos in yeast strain MaV203, testing activation of the His promoter by growth on –His/3-AT plates. Constructs 1 and 3 (full length and 1-726), which incorporate the HR region, support growth on these plates. Constructs missing the HR region (2 and 5) do not. Thus, the HR domain of KLC-3 likely mediates interaction with c-Mos.

Fig. 20



DISCUSSION

I: SUMMARY OF MAJOR FINDINGS AND CONCLUSIONS

I.a: KLC-3 is a Novel KLC

It has been shown by a PCR and sequence based strategy that KLC-3 is the gene product of a putative gene in the ERCC2 locus, as proposed previously (Lamerdin et al, 1997; Rahman et al, 1998). As these groups also conclude, this gene is a KLC family member. In line with these proposals, it was shown by sequence alignments that KLC-3 has high homology to other KLC family members, and that this homology is constrained by typical KLC patterns: divergent sequences in the N-terminus, C-terminus and PEST regions, conserved sequences in the HR and TPR motifs, and conserved PKC phosphorylation sites (Wedaman et al., 1993; Gindhart, Jr. and Goldstein, 1996; Gauger and Goldstein, 1993; Diefenbach et al., 1998; Cyr et al., 1991; Beushausen et al., 1993). Furthermore, it was shown that KLC-3 binds to KHC isoform Kif5c *in vitro*, and that this is mediated by the HR motif, which mediates KHC interaction for other KLC family members (Verhey et al., 1998; Gauger and Goldstein, 1993; Diefenbach et al., 1998). Finally, it was demonstrated that KLC-3 binds to microtubules in a nucleotide sensitive manner, consistent with kinesin family paradigms (Brady, 1985; Vale et al., 1985a; Bloom and Endow, 1994). In conjunction, these results indicate that KLC-3 is a member of the KLC family.

I.b: KLC-3 has unique characteristics *in vivo*

BLAST searches and RT-PCR together demonstrate that KLC-3 is expressed both during early development and subsequently in a wide variety of rodent tissues. The RT-PCR analysis further demonstrated that KLC genes differ in their expression patterns in sperm development: KLC 1 and 2 are expressed in spermatocytes, whereas KLC-3 is expressed predominantly in spermatids. Furthermore, both Kif5c and 5a -both purportedly neuronal kinesins (Niclas et al., 1994; Nakagawa et al., 1997; Xia et al., 1998) – were detected in the testes. In addition, it was shown that all three Kif5 genes are expressed in spermatocytes, whereas only Kif5c is expressed in spermatids. It is concluded that kinesin gene expression differs by cell type and is developmentally regulated. Thus, developing cells only employ a subset of the possible KHC:KLC combinations.

The RT-PCR analysis indicated that KLC-3 could interact only with Kif5c in spermatids, as no Kif5a or b was detected in these cells. Western analysis confirmed higher KLC-3 expression levels in spermatids, and lower Kif5c expression, suggesting an excess of light chain versus heavy chain in spermatids. Finally, immunofluorescence localized KLC-3 to sperm tails, whereas no Kif5c was detected here. In conjunction, these three lines of evidence indicate that KLC-3 does not interact with any known KHC in sperm tails. It either interacts with a novel KHC here or serves a unique and novel function. These results are consistent with localization of KLC-3 to ODFs, with only minor localization to the axoneme, as determined previously by immunoelectron microscopy (van der Hoorn, per coms). In line with these observations, KLC-3 was found to interact with ODF-1 and SPAG4, both of which localize to ODFs (Shao et al.,

1999; Schalles et al., 1998). Furthermore, the interaction was shown to be mediated by leucine zippers, in line with ODF protein interaction patterns (Shao and van der Hoorn, 1996; Shao et al., 1997). The fact that co-localization and yeast two hybrid interaction results support each other suggests that this interaction may be biologically significant. It is proposed that KLC-3 has a function in sperm tails that is unrelated to MT based transport.

I.c: KLC-3 interacts with the c-Mos extracatalytic domain

Finally, KLC and Mos interactions were investigated. The interaction of Mos and KLC-3 in yeast two-hybrid had previously been confirmed by using immunoprecipitation techniques Tarnasky et al. I therefore mapped the interacting domains using yeast two hybrid, and show that the HR region of KLC-3 interacts with the extracatalytic domain of c-Mos. This is consistent with a failure of c-Mos to phosphorylate KLC *in* vitro (van der Hoorn, per coms). This is significant because it suggests that KLC-3 is not a substrate, but rather that the interaction fulfills some other function. KLC-3 does not localize to MTs in the testes, but could do so in other tissues. One possibility is that KLC-3 could function as an MT tether for c-Mos in these tissues. The function of the KLC-3/c-Mos interaction is not clear at this time.

II: CRITIQUE OF CONCLUSIONS

The conclusions drawn above are subject to various flaws and weaknesses, which require discussion: here the major experimental findings are challenged and either

defended or refuted, and suggestions for experimental strategies to resolve the main issues are made.

II.a: KLC-3 is a novel KLC family member

The cloning of KLC-3 and its localization to the ERCC2 locus is difficult to refute. The PCR analysis shows that KLC-3 and ERCC2 sequences are linked as predicted, and sequence alignments show that KLC-3 is the product of the ERCC2 locus gene. What can be challenged is the classification of KLC-3 into the KLC family. For example, the KLC-3 sequence has only one N-terminal methioinine. No alternative splice sites were demonstrated at the C-terminus and no evidence was shown demonstrating the existence of multiple splice variants. Additionally, KLC-3 is missing the 6^{th} TPR repeat, and is lacking conserved tyrosine kinase phosphorylation sites. Furthermore, KLC-3 sequences are less conserved with rodent KLCs than those of lower eukaryotes such as *C.elegans*. As discussed in subsequent sections, these sequence divergences are interpreted as minor but significant. Further, it is suggested that they generate functional diversity in the KLC family, rather than excluding KLC-3 from it.

Another issue is that KLC-3 and KHC interaction was not demonstrated *in vivo*. This underlies the traditional definition of a KLC: it is a peptide that co-purifies with heavy chains (Vale et al., 1985a) in a 1:1 stoichiometric ratio, and exists in cells as a tetramer (Bloom et al., 1988). Although it is demonstrated Kif5c:KLC-3 interaction *in vitro*, it was shown that this interaction may not occur for a pool of KLC-3 in spermatids. Some KLC-3 may interact with Kif5c in spermatids, however this may be difficult to demonstrate as immunoprecipitation experiments are not possible due to the insoluble

nature of sperm tails. However, the conservation of sequence and *in vitro* function cannot be explained as a coincidence or an artifact. Additionally, the historical co-purification of KLC peptides with KHC peptides does not preclude other functions and interactions for these peptides. Thus, the classification of KLC-3 in the KLC familyis favoured. In future experiments, it will be interesting to determine if KLC-3:KHC complexes exist in other tissues. Co-localization, co-fractionation or ideally co-immunoprecipitation with a KHC isoform from other rodent tissues such as brain or ovary could be investigated.

II.b: KCL-3 has a novel and unique function *in vivo*

Our conclusion that KLC-3 and Kif5c do not interact in spermatids is based on two findings: differences in expression levels and lack of co-localization. Both can be challenged: molecular biology techniques are often used for quantitation, however generally this is confined to self: in other words, comparison of KLC-3 expression to KHC expression by Western or PCR is problematic. Numerous complicating variables confound indirect comparison, such as differences in primer efficiency, mRNA/protein stability, antibody affinity, etc. The techniques are therefore not rigorous enough to allow conclusions regarding the exact stochiometry of the two genes in spermatids. Furthermore, quantitation relative to self can also be problematic, especially by PCR. PCR amplification is subject to many variables, and minor tissue specific differences (such as competing reactions) can result in large discrepancies due to the exponential nature of PCR amplification. The ECL Western technique used by us is also subject to non-linear amplifications: the signal generated by the conjugated enzyme (HRP) follows

exponential kinetics (Robbins, 2000). Rigorous determination of relative KLC-3 and KHC stoichiometry can only be achieved by analytical chemistry techniques, such as a purification scheme (HPLC or other) coupled to a quantitation scheme (Mass Spectroscopy, Atomic Absorption Spectroscopy, etc.). Our justification for the interpretation of the RT-PCR results as quantitative is presented in the results section. Based on that logic, it is suggested that PCR quantitation relative to self may hold some validity in this case. The corroboration of Western and PCR results with both self and KLC-3 vs. KHC quantitation further supports our proposal. Furthermore, the relative intensity of KLC-3 and KHC signals in immunofluorescensce are also consistent with low KHC expression in spermatids. Finally, the lack of KLC-3 and KHC co-localization implies a lack of interaction irrespective of stoichiometric ratios. Thus, it is proposed that our conclusions are accurate to a reasonable (non linear) approximation, and do not deem further quantitation necessary.

The immunolocalisation experiments may also be questioned. For example, the KLC-3 monoclonal antibody specificity can be questioned on the basis of its weak affinity for KLC-3 in Westerns (van der Hoorn, per coms). However, the specificity of this antibody has additionally been demonstrated by both immunoprecipitations (van der Hoorn, per coms). Furthermore, the antibody stained sperm tails in both immunofluorescence and EM sections. Based on this reasoning, it is concluded that the sperm tail signal generated by the 11g6b2 antibody is specific for KLC-3. It is also possible that KLC-3 and KHC antigens were inaccessible for immunoreactivity. For KLC-3, polyclonal antibodies and monoclonal antibodies both generated similar staining patterns in the testis, arguing against this possibility. Similarly, three different

monoclonals generated the same pattern of KHC staining. It is thus probable that the majority of KLC-3 and KHC was detected. However, it is possible that faint signals or low levels of KLC-3 or KHC were not detected. This could be pursued in the future by fractionation experiments coupled to immunobloting. The spermatid/mature sperm can be separated into several biochemical/cytoskeletal fractions by extraction/centrifugation techniques (Oko, 1988; Oko and Clermont, 1988). Thus, the co-localization of KLC-3 and KHC could be assayed more precisely by this technique.

The third piece of evidence suggesting a novel function for KLC-3 was the yeast two-hybrid result demonstrating interaction with ODF proteins. This system can generate both false positives and negatives. Thus, interpretation of results obtained by this technique requires corroboration by another technique and demonstration of biological relevance. Biological relevance for the yeast two-hybrid results has been demonstrated both in this study, and previously in our lab (van der Hoorn, per coms), by the localization of KLC-3 to sperm tails. The agreement of immunolocalisation and yeast two hybrid results, together with the discovery of a biologically relevant interacting motif, is statistically and intuitively unlikely to be the product of artifact co-incidence. Although Spag4 and KLC-3 failed to immunoprecipitate, the proteins may well interact in another buffer or assay (affinity columns, co-localization in cell transfections, etc). It is also proposed that other ODF proteins be analyzed for KLC-3 binding. Although these have not been cloned, ODFs can be biochemically isolated (Oko, 1988; Oko and Clermont, 1989) and tested for KLC-3 binding activity *in vitro*. The simplest method might be the separation of ODF proteins by SDS-PAGE electrophoresis, followed by blot overlay assays with *in vitro* translated or bacterially expressed KLC-3. Since molecular

weights of the major ODF components have been characterized by electrophoresis (Oko, 1988), such an experiment could be quite informative. In conclusion, it is suggested that the KLC-3 /ODF protein interaction is a reasonable hypothesis, and furthermore it is proposed that KLC-3 may additionally interact with other ODF proteins.

II.c: Mos/KLC interactions

The Mos KLC-3 deletion studies could also be subject to yeast two hybrid artifacts. Positive controls were not available for any deletion constructs, hence false negatives cannot be ruled out. It is unlikely that the entire region between nucleotides 230-885 of c-Mos interacts with KLC-3. However, the areas outside this region are clearly not required for the interaction. Furthermore, precise mapping of the interacting amino acids was not the goal of this project. Rather, the aim was to delineate a larger region that could then be targeted by random mutagenesis to disrupt the Mos-KLC interaction. Such mutants can then be used to determine the functional significance of KLC-3/c-Mos interaction (see below, III.a.i). Thus, further work with deletion constructs is not likely to be informative in the context of c-Mos/KLC-3 functional significance.

It is still a theoretical possibility that c-Mos phosphorylates KLC-3. Although previous experiments in our lab did not detect phosphorylation *in vitro*, (van der Hoorn, per coms) no positive controls were used and furthermore *in vitro* does not necessarily reflect *in vivo*, as has been demonstrated previously for KLCs by Hollenbeck, 1993). *In vivo* (co-translation) experiments did not detect increased phosphorylation of KLC-3 by c-Mos, but this could well be a consequence of the fact that KLCs are already phosphorylated *in vivo* at various sites (Sato-Yoshitake et al., 1992; McIlvain, Jr. et al.,

1994; Matthies et al., 1993; Lindesmith et al., 1997b; Lee and Hollenbeck, 1995; Hollenbeck, 1993). Analogous co-transfections coupled to phosphopeptide mapping could resolve this question. Furthermore, the hypotheses of tethering vs. substrate are not mutually exclusive. In light of the above evidence, it is suggested that the tethering hypothesis is more attractive. The existence of c-Mos:KLC-3:KHC:MT complexes should be investigated, perhaps by coupling immunofluorescence, cell fractionation/immunoblotting and immunoprecipitation studies both in co-transfections in tissue culture and from relevant rodent tissues.

Finally, the failure of the study on KLC family members and c-Mos interaction suggests attacking this problem from a different angle: biological significance. Results of immunoprecipitations done in our lab (van der Hoorn, per coms) suggest that KLC-1 and 2 likely do not complex with c-Mos in the testis. Therefore, prior to investigating this interaction any further *in vitro*, attempts should be made to determine if the complexes exist in other cell types expressing c-Mos (such as oocytes). This could, for example, be done simultaneously with investigations on c-Mos/KLC-3 complexes and their potential localization to MTs as suggested above.

III. IMPLICATIONS OF FINDINGS AND SPECULATION

III.a: Unusual Kinesin Functions

i) Tethering of cell regulatory molecules to MTs

Our observation that KLC-3 binds the extracatalytic domain of c-Mos raises the possibility that KLC-3 is not a substrate for c-Mos. Based on reports that KRPs such as *Pavarotti* (Carmena et al., 1998; Lee et al., 1995; Adams et al., 1998), COS2 (Robbins et

al., 1997; Sisson et al., 1997) and the Kif3 superfamily (Hanlon et al., 1997) can tether signaling molecules to the MT lattice, it is interesting to speculate that KLC-3 fulfills a similar function for c-Mos. Interesting parallels can be drawn to the above studies. For example, COS2 binds to MTs but does so in an ATP independent manner, which suggests that COS2 may not function as a motor but rather as a tether(Sisson et al., 1997). Similarly, no motor associated function for KLC-3 in spermatids was found. Instead, it has been proposed that KLC-3 may associate with ODFs. In parallel with this observation, c-Mos has been determined to exist in insoluble complexes in cultured rat sperm cells (Nagao, 1995a). Thus, it is possible that KLC-3 could tether c-Mos to ODF fibers or other insoluble structures. However, ODF expression is confined to spermatids, whereas KLC-3 expression has been detected here within the brain, ovary and other cell types. Based on our *in vitro* results, coupled to preliminary observations that KLC-3 localizes to process similar to neuronal axons/dendrites in the brain (Demetrik, 1999), it is hypothesized that KLC-3 may fulfill a more conventional functions in these cell types (ie. interact with KHC/MTs).

Paralleling these observations are the reports that Mos (in both oocytes and transformed cells) localizes to MTs, phosphorylates tubulin and potentially regulates MT dynamics (Singh and Arlinghaus, 1997; Bai et al., 1992; Zhou et al., 1991a; Zhou et al., 1991b; Rulong et al., 1995; Fukasawa and Vande Woude, 1995; Bai et al., 1992; Rulong et al., 1995; Wu et al., 1997; Verlhac et al., 1996). Thus, our hypothesis that KLC-3 functions as a MT tether for c-Mos may explain a wide number of observations and have great functional significance. Our lab has already demonstrated that c-Mos partitions to taxol-stabilized microtubules isolated in the presence of AMP-PNP. This assay suggests

that c-Mos binds to either kinesins co purifying with the MTs or the MTs themselves. In order to discriminate between these possibilities, it will be necessary to determine binding based on purified components (ie MTs +/- kinesin). The interaction of c-Mos with other KLC genes may also need to be revisited. Additionally, it is warranted to study the expression and localization of KLC-3 and other kinesins in the oocyte, and the potential association of c-Mos with kinesins in these cells. Finally, the significance of c-Mos localization to MTs in c-Mos induced cell transformation will be investigated. We have proposed to study the effects of disrupting c-Mos localization to MTs on cellular transformation by generating mutations of both c-Mos and KLC-3. Our characterization of KLC-3 and c-Mos interacting domains will facilitate the generation of these mutants.

ii) Interaction of KLC-3 with ODFs and Localization to Sperm Flagella

Our localization of KLC-3 to sperm tails in the absence of known KHC genes suggests either that KLC-3 interacts with a novel KHC here or that it serves an unusual function. Our observation that KLC-3 interacts with ODF proteins via its leucine zipper - a common theme for ODF structural proteins (Shao and van der Hoorn, 1996; Shao et al., 1997; Shao et al., 1999)- and the subsequent localization of the bulk of KLC-3 to ODFs and mitochondria rather than MTs (van der Hoorn, per coms) support the latter hypothesis. It is interesting to note that KLCs have two independent protein interaction motifs: the HR and TPR motifs (Cyr et al., 1991). Rotary shadowed EM pictures suggest a fan tail structure for the molecule, and based on these two observations it has been suggested that KLCs are structurally well adapted as linker molecules (Hisanaga et al., 1989; Hirokawa et al., 1989; Cyr et al., 1991). It is thus possible that KLC-3 acts as a linker of sorts to tether mitochondria to the ODFs. Based on our deletion analysis, it is

hypothesized that mitochondria interact with either the TPR or the C-terminal sequences of KLC-3 and ODFs interact with the HR/leucine zipper. This hypothesis can be tested by biochemical fractionation studies: it is possible to isolate spermatid specific mitochondria (Burgos et al., 1995). Subsequently, either the co-fractionation or *in vitro* interaction of KLC-3 and deletion constructs with these could be analyzed by immunoblotting or other techniques.

As noted above, some KLC-3 label on the flagellar axonemal MTs has been observed (van der Hoorn, per coms). This observation suggests a more conventional kinesin function for KLC-3, in addition to possible ODF binding. The recent observations that KRPs are important in intraflagellar transport of flagellar components in *Chlamydomonous* (Cole et al., 1998) may have parallels in higher eukaryotic structures such as sperm flagella (Henson et al., 1997). It is possible that KLC-3 interacts with a novel KHC in these structures. However, this is not supported by chromosomal localization analysis of the mouse, which revealed only the 3 KHC genes analyzed here (Kif5a, b and c) (Xia et al., 1998). The anticipated completion of various genome projects may result in the identification of a novel KHC gene. Another, unprecedented, possibility would be the interaction of KLC-3 with a KRP. Some KRPs are known to interact with peptides with putatively analogous functions to KLCs in mice (Yamazaki et al., 1995) and the same KRPs localize to sperm flagella in lower eukaryotes (Henson et al., 1997) and rodents (Miller et al., 1999). Current attempts at repeating the KLC-3 immuno-EM with polyclonal antibodies should improve our understanding of KLC-3 localization to ODFs and MTs, as initial results suffered from low signal. These can be used as a basis for further predictions. Furthermore, the development of these same polyclonals should

hasten our understanding of KLC-3 partitioning to spermatid cytoskeletal structures, as these can be biochemically isolated (Oko, 1988; Oko and Clermont, 1988) and the fractions analyzed by immunoblotting. The generation of KLC-3 knock out mice (ongoing work in our lab) should also provide insight to the role of KLC-3 in spermatids.

iii) Kif 5c and putative manchette localization

Our immunolocalisation attempts with KHC antibodies demonstrated the localization to punctate, vesicular structures of unknown identity. Based on previous reports of KHC immunolocalisation to the manchette of spermatids using the same antibodies, and the similarity of our and their immunoblots (Hall et al., 1992), it is reasonable to speculate that some of these structures could represent manchettes. Manchettes are transient structures expressed only in spermatids (Soley, 1997; Rattner and Brinkley, 1972). However, we have failed to detect KLC-1 or 2 in spermatids, and detected KLC-3 only in sperm tails. Based on these observations, in conjunction with the published report, three possibilities arise: a) Kif5c may not associate with any KLC in the manchette b) Kif5c may interact with KLC-3 in the manchette, which was not detected, or c) Kif5c may interact with a novel KLC in the manchette. The first possibility is especially intriguing based on our analogous hypothesis for KLC-3 in sperm tails, and the unusual and poorly characterized function of the manchette (Soley, 1997; Russell et al., 1991). The reader is refered reader back to the introduction and discussions on fungal KHCs (which lack KLCs) and observations of KLC independent function of KHC from higher eukaryotes *in vitro*. It is thus possible that the functions of KLCs are dispensable for KHC in the manchette. The second possibility is being addressed by the renewed immuno-EM study as noted above. The third correlates with our detection of KLC

homologous sequences on human chromosome 6. Also suggestive is the detection of a weak FISH signal on human chromosomes 4, 11 and 16 while localizing KLC-1 (Goedert et al., 1996). The localization to chromosome 11 likely represents KLC-2 (Rahman et al., 1998), however the signal of 4 and 16 could represent either background or novel KLC genes. The fact that KLC-3 signal was not detected (chromosome 19) is consistent with its sequence dissimilarity to KLC-1, argues against background, and supports the notion of novel KLC genes at these loci. Additional evidence comes from reports of KLC genes with molecular weights of aprox. 72kDa from bovine brain (Matthies et al., 1993), too large to be either KLC-1 (61kDa), or KLC-3 (56kDa), and probably also too large to be KLC-2 (67kDa) (although it could be a larger splice variant). Completion of the human genome project should determine the veracity of these speculations.

III.b: Functional Diversity of Kinesins

i) KLC genes and animal complexity

Speculation on the number of KLC and KHC genes expressed by rodents evokes the hypothesis elaborated in the introduction: namely that KLC genes have evolved to generate additional plasticity of kinesin function. It is concluded based on the dendograms of KLC genes and the BLAST searches that KLC-2 and 3 genes are only represented in higher animals, while KLC-1 is likely expressed in all eukaryotes except the fungi (and such). Thus kinesin gene number correlates with animal complexity, as suggested in the introduction. Although KLC-2 has only been detected in mice, the FISH study mentioned above (Goedert et al., 1996) and our own BLAST searches both produced hits on chromosome 11 in humans, which is syntenic with the mouse KLC-2

gene (Rahman et al., 1998). This argues for the presence of KLC-2 in humans. Furthermore, the detection of the KLC-3 gene sequence in the ERCC-2 locus of mice, hamster and human argues for the presence of KLC-3 in primates. This is supported by observations of KLC-3 immunoreactivity in bovine and rhesus monkey sperm tails (Sutovsky, 1999).

The fact that KLC-3 sequences are less similar to mouse KLC-1 than the *C. elegans* and *Drosophila* KLC-1 are to rat KLC-1 is interpreted by us as a requirement by higher organisms for increased KLC diversity. It is theorized that KLC-3 sequence idiosyncrasies may underlie specificity with regards to KHC interaction, cargo binding, regulation by kinases, regulation of KHC function, dimerisation specificity (ie itself vs. other KLCs), and the novel/proposed functions suggested for KLC-3 in sperm tails and c-Mos tethering. The various experiments suggested in other sections could be coupled to controls using KLC-1 and 2 genes to determine if these speculations are based in reality.

ii) Kinesin Expression is developmentally regulated and cell type specific

The rationale presented above is supported by our observations of differences in the expression patterns of both KHC and KLC genes. Similar observations of expression specificity have been made for KLC genes in neuronal cells (Rahman et al., 1999; Rahman et al., 1998), KHC genes in developing (Vignali et al., 1996; Vignali et al., 1997) and differentiated neuronal cells (Niclas et al., 1994) and KLC-1 splice isoforms amongst various tissues (Su et al., 1997; Beushausen et al., 1993). Thus, distinct cell types likely express different subsets of kinesin genes, and/or express these genes to different extents. It is surmised that this is a consequence of specialized needs of the differentiated cell types and functional variance amongst the individual kinesin gene

products. Furthermore, it is suggested that KLC gene duplication is not a consequence of the need for redundancy.

Current testing of this hypothesis by the generation of KLC-3 knockout mice is ongoing. Based on our observations of high expression in spermatids and oocytes, it is theorized that phenotypic defects will predominantly be associated with spermatids and potentially oocytes. This is consistent with a observations of neuro-muscular defects in KLC-1 knock-out mice (Rahman et al., 1999), which is a neuronally enriched KLC (Rahman et al., 1998).

III.c: KLC-3 as a regulator of KHC function

i) KLC-3 as a regulatory target

KLCs have been widely theorized (Hollenbeck, 1993; Cyr et al., 1991) and more recently implicated (Verhey et al., 1998; Lindesmith et al., 1997a) as regulators of kinesin function as suggested above in the discussion (II.c), potential phosphorylation of KLC-3 by c-Mos has not been ruled out, and experiments to test this hypothesis have been suggested. Alternatively, it is also possible that c-Mos regulates KLC-3/kinesin activity by mechanisms not involving phosphorylation. Precedent for this is evident from studies demonstrating that native calmodulin partitions to MTs in an AMP-PNP assay in classical kinesin fashion, binds to KLCs but not KHC in vitro, and this binding induces changes in MT stimulated kinesin ATPase activity (Matthies et al., 1993). Assays such as those used in this report could determine whether c-Mos binding to KLC-3 serves such a function. It is also interesting to note that KLC-3 does not have several tyrosine kinase sites, which are conserved in KLC-2 (this study) and KLC-1 homologues, and are

putative regulatory targets (Cabeza-Arvelaiz et al., 1993). This suggests potential differences in regulatory mechanisms controlling KLC-3 vs. KLC-1 and 2.

III.d: Kinesins and cargo transport

Some indirect evidence and speculations for KLC-3 as a linker molecule between ODFs and mitochondria in spermatids (refer to section III.c.ii) are discussed. It was also suggested that KLC-3 may function as a more conventional KLC in other cell types such as neurons, (based on preliminary experiments suggesting immunolocalisation to these cells) and juxtaposition to paradigms of kinesin function in axonal transport (Bloom and Endow, 1994; Goldstein and Philp, 1999). In conjunction, these observations and suggestions raise the possibility that KLC-3 mediates mitochondrial transport in other cell types. Mitochondrial targeting of specific KLC isoforms of kinesin has been described previously (Khodjakov et al., 1998), and KHC has been linked to mitochondrial transport in various cell types (reviewed in Goldstein and Philp, 1999). Cellular fractionation experiments coupled with immunoblotting, immunolocalisation of KLC-3 in various tissues, and localization of GFP-KLC-3 constructs in cell lines co-transfected with KHC and KLC-3 (excess KLC has been reported to be insoluble in transfected cells, suggesting KHC co-transfection is necessary (Diefenbach et al., 1998)) could be conducted to determine KLC-3 interaction with MBOs.

Immunolocalisation experiments with three separate KHC antibodies resulted in a punctate, granular staining pattern in testes. Previous reports have localized KHC to the manchette (Hall et al., 1992) (see III.a.iii above) and to the Golgi (Johnson et al., 1996a). Based on the various patterns this staining displayed at higher magnifications, it is suggested that both may have been observed. Additionally, preliminary experiments

revealed staining of vesicular structures reminiscent of Golgi in brain cryosections probed with H1 and H2 antibodies, in line with published reports. Our results of vesicular staining patterns with KHC are consistent with paradigms of KHC function.

III.e: The Testis as a Model System

In light of the evidence presented, it is believed that the utility of the testis as a model system for the study of kinesins has been demonstrated. This report has raised interesting possibilities for novel and unconventional kinesin functions, has detected expression of purportedly neuronal genes in the testis, lends support to the concepts of kinesin functional diversity and developmental/tissue specific regulation, and has generated interesting tangents such as the c-Mos/KLC-3 interaction. It is believed these topics are worthy of further investigation, and thus experiments to pursue these ideas have been suggested.

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