Abstract:
The present invention relates to methods and pharmaceutical compositions for enhancing myelination in a subject in need thereof. In particular, the present invention relates to a method of enhancing myelination in a subject in need thereof comprising administering the subject with a therapeutically effective amount of an inhibitor of the Crb3-HPO-YAP signaling pathway.
METHODS AND PHARMACEUTICAL COMPOSITIONS FOR ENHANCING MYELINATION

FIELD OF THE INVENTION:
The present invention relates to methods and pharmaceutical compositions for enhancing myelination in a subject in need thereof.

BACKGROUND OF THE INVENTION:
Myelin is a vital component of the central and peripheral nervous system. The systematic wrapping of an axon by insulating myelin sheaths is a remarkable event in the development of the vertebrate central nervous system. Consisting of 70% lipid and 30% protein, myelin is formed both by oligodendrocytes (OLs) in the central nervous system (CNS) and by Schwann cells in the peripheral nervous system (PNS). Working as insulation, myelin enhances the speed and integrity of nerve signal propagation down the neural axon, allowing signals to pass back and forth between the brain and the nerves of the periphery over long distances. Damage to the myelin sheath can lead to a variety of neurological disorders with often devastating consequences.

Previous studies have shown that myelination is a multistep process in which a myelinating cell adheres to an axon, then ensheaths and wraps it, culminating with exclusion of the cytoplasm from the spiraling processes to form compact myelin. The myelin sheath is formed by the plasma membrane, or plasmalemma, of glial cells: oligodendrocytes in the CNS, Schwann cells in the PNS. The plasmalemma makes contact with the axon and then begins to wrap around it, spiral fashion, the inner mesaxon continuing to circle the axon as the plasmalemma grows and flattens, squeezing out most of the cytoplasm, until the end result is a laminated sheath consisting of multiple concentric lamellae formed of plasma membrane, each lamella consisting of a total of four lipid leaflets. The myelin sheath is formed in segments along the length of the axon.

Fast nerve conduction relies on the electrical isolation of axons via successive myelin segments. The myelin geometry - diameter and internodal length - is therefore a critical parameter of the nerve conduction velocity. Indeed in the peripheral nervous system, mutations that induce thinner or shorter myelin sheaths strongly affect nerve conduction leading to peripheral neuropathies. Myelin geometry is strictly controlled in myelinating cells and all myelin segments on the same axon have similar diameter and internodal length. The
molecular mechanisms that are responsible for the homogeneity of myelin thickness in Schwann cells have recently been uncovered. Although internodal length strongly correlates with nerve and limb elongation, the molecular mechanisms of myelin elongation remain unknown. The present invention addresses means of modulating myelin elongation in a subject in need thereof.

**SUMMARY OF THE INVENTION:**

The present invention relates to methods and pharmaceutical compositions for enhancing myelination in a subject in need thereof. In particular, the present invention is defined by the claims.

**DETAILED DESCRIPTION OF THE INVENTION:**

The inventors show here that controlled myelin elongation in Schwann cells is dynamically regulated by the stimulation of YAP transcription cofactor activity during axonal elongation and, conversely, limited by the inhibition of YAP activity via the polarity protein Crb3 and the HPO pathway. YAP promotes myelin gene transcription while the polarity protein Crb3, localized at the tips of the myelin sheath, activates the HPO pathway to temper YAP activity, allowing for optimal longitudinal growth of adjacent myelin segments. Dystrophic Dy2j/2j mice that mimic a human peripheral neuropathy with reduced internodal lengths have decreased nuclear YAP which, when corrected, leads to longer internodes. The inventors propose that defects in YAP activation underlie peripheral neuropathies with reduced internodal length and the modulation of the HPO/YAP pathway may be used to correct myelination and myelin geometry.

The present invention relates to a method of enhancing myelination in a subject in need thereof comprising administering the subject with a therapeutically effective amount of an inhibitor of the Crb3-HPO-YAP signaling pathway.

In particular the method of the present invention is particularly suitable for correcting myelination and myelin geometry. More particularly the method of the present invention is suitable for enhancing and promoting myelin elongation.

The method of the present invention has wide applicability to the treatment or prophylaxis of disorders affecting the regulation of peripheral nerves (i.e. peripheral...
neuropathies), including peripheral ganglionic neurons, sympathetic, sensory neurons, and motor neurons. In particular, the method of the present invention is useful in treatments designed to rescue, for example, retinal ganglia, inner ear and accoustical nerves, and motoneurons. The method of the present invention is particularly suitable for the treatment of peripheral demyelinating neuropathies. The wide variety of morphologies exhibited by peripheral neuropathies can each be uniquely attributed to an equally wide variety of causes. For instance, peripheral neuropathies can be genetically acquired, can result from a systemic disease, or can be induced by a toxic agent or an infectious agent.

In some embodiments, the method of the present invention is suitable for the treatment of hereditary peripheral neuropathies. Hereditary neuropathies are caused by genetic abnormalities which are transmitted from generation to generation. For several of these, the genetic defect is known, and tests are available for diagnosis and prenatal counseling. In particular, the diagnosis of a hereditary neuropathy is usually suggested with the early onset of neuropathic symptoms, especially when a positive family history is also present. Prior to the recent genetic advances, the diagnosis was supported by typical findings of marked slowing of the nerve conduction studies on electromyography and a nerve biopsy. Typical findings on a nerve biopsy include the presence of so-called onion-bulbs, indicating a recurring demyelinating and remyelinating of the nerve fibers. There are a number of hereditary demyelinating neuropathies. Examples include but are not limited to Refsum's disease, Abetalipoproteinemia, Tangier disease, Krabbe's disease, Metachromatic leukodystrophy, Fabry's disease, Dejerine-Sottas syndrome, - and others. Of all the hereditary peripheral neuropathies, the most common by far is Charcot-Marie-Tooth Diseases. Charcot-Marie-Tooth (CMT) Diseases are the most common hereditary neurological disorders. It is characterized by weakness and atrophy of muscles due to segmental demyelination of peripheral nerves and associated degeneration of axons and anterior horn cells. During the last 15 years, there has been a substantive increase in knowledge about the genetic basis of Charcot-Marie-Tooth disease (CMT) with over 60 genes known at present. A regularly updated list can be found at http://www.molgen.ua.ac.be/CMTMutations/Home/IPN.cfm. Autosomal dominant inheritance is usual, and associated degenerative CNS disorders, such as Friedreich's ataxia, are common. In some embodiments, the method of the present invention can be used for the treatment of Charcot-Marie-Tooth disease type 4F. In some embodiments, the method of the present invention can be used to treat, or at least reduce the severity of Amyotrophic lateral sclerosis (ALS). In some embodiments, the method of the present
invention can be used in the treatment of Familial Amyloidotic Neuropathy and other related hereditary neuropathies. The method of the present invention can be used in the treatment of hereditary porphyria, which can have components of peripheral neuropathy. Still another hereditary neuropathy for which the method of the present inventions can be used for treatment is hereditary sensory neuropathy Type II (HSN II). In some embodiments, the method of the present invention can be used for the treatment of certain muscular dystrophies. In some embodiments, the method of the present invention can be used for the treatment of congenital muscular dystrophy IA.

The method of the present invention is also suitable the treatment and maintenance of acquired neuropathies.

In some embodiments, the method of the present invention is suitable for the treatment of diabetic neuropathies. Diabetes is the most common known cause of neuropathy. It produces symptoms in approximately 10% of people with diabetes. In most cases, the neuropathy is predominantly sensory, with pain and sensory loss in the hands and feet. But some diabetics have mononeuritis or mononeuritis multiplex which causes weakness in one or more nerves, or lumbosacral plexopathy or amyotrophy which causes weakness in the legs.

In some embodiments, the method of the present invention can also be used in the treatment of immune-mediated neuropathies. The main function of the immune system is to protect the body against infectious organisms which enter from outside. In some cases, however the immune system turns against the body and causes autoimmune disease. The immune system consists of several types of white blood cells, including T-lymphocytes, which also regulate the immune response; and B-lymphocytes or plasma cells, which secrete specialized proteins called "antibodies" Sometimes, for unknown reasons, the immune system mistakenly attacks parts of the body such as the peripheral nerves. This is "autoimmune" Peripheral Neuropathy. There are several different types, depending on the part of the peripheral nerve which is attacked and the type of the immune reaction. For instance, a inhibitor of the Crb3-HPO-YAP signaling pathway can be used to treat Guillain-Barre Syndrome (GBS). An acute neuropathy because it comes on suddenly or rapidly. Guillain-Barre Syndrome can progress to paralysis and respiratory failure within days or weeks after onset. The neuropathy is caused when the immune system destroys the myelin sheaths of the motor and sensory nerves. It is often preceded by infection, vaccination or trauma, and that is
thought to be what triggers the autoimmune reaction. The disease is self-limiting, with spontaneous recovery within six to eight weeks. But the recovery is often incomplete.

Other neuropathies which begin acutely, and which can be treated by the method of the present invention, include Acute Motor Neuropathy, Acute Sensory Neuropathy, and Acute Autonomic Neuropathy, in which there is an immune attack against the motor, sensory or autonomic nerves, respectively. The Miller-Fisher Syndrome is another variant in which there is paralysis of eye gaze, incoordination, and unsteady gait. Still another acquired neuropathy which is may be treated by the method of the present invention is Chronic Inflammatory Demyelinating Polyneuropathy (CIDP). CIDP is thought to be a chronic and more indolent form of the Guillain-Barre Syndrome. The disease progresses either with repeated attacks, called relapses, or in a stepwise or steady fashion. As in GBS, there appears to be destruction of the myelin sheath by antibodies and T-lymphocytes. But since there is no specific test for CIDP, the diagnosis is based on the clinical and laboratory characteristics.

Chronic Polyneuropathies with antibodies to peripheral nerves is still another peripheral neuropathy for which the method of the present inventions can be employed to treat or prevent. In some types of chronic neuropathies, antibodies to specific components of nerve have been identified. These include demyelinating neuropathy associated with antibodies to the Myelin Associated Glycoprotein (MAG), motor neuropathy associated with antibodies to the gangliosides GM1 or GD1a, and sensory neuropathy associated with anti-sulfatide or GDlb ganglioside antibodies. The antibodies in these cases bind to oligosaccharide or sugar like molecules, which are linked to proteins (glycoproteins) or lipids (glycolipids or gangliosides) in the nerves. It is suspected that these antibodies may be responsible for the neuropathies.

The method of the present invention can also be used as part of a therapeutic plan for treating neuropathies associated with vasculitis or inflammation of the blood vessels in peripheral nerves. Neuropathy can also be caused by Vasculitis - an inflammation of the blood vessels in peripheral nerve. It produces small "strokes" along the course of the peripheral nerves, and may be restricted to the nerves or it may be generalized, include a skin rash, or involve other organs. Several rheumatological diseases like Rheumatoid Arthritis, Lupus, Periarteritis Nodosa, or Sjogren's Syndrome, are associated with generalized Vasculitis, which can also involve the peripheral nerves. Vasculitis can cause Polyneuritis,
Mononeuritis, or Mononeuritis Multiplex, depending on the distribution and severity of the lesions.

In some embodiments, the method of the present invention is suitable for the treatment of neuropathies associated with monoclonal gammopathies. In Monoclonal Gammopathy, single clones of B-cells or plasma cells in the bone marrow or lymphoid organs expand to form benign or malignant tumors and secrete antibodies. "Monoclonal" is because there are single clones of antibodies. And "Gammopathy" stands for gammaglobulins, which is another name for antibodies. In some cases, the antibodies react with nerve components; in others, fragments of the antibodies form amyloid deposits.

In some embodiments, the method of the present invention is suitable for the treatment of neuropathies associated with tumors or neoplasms. Neuropathy can be due to direct infiltration of nerves by tumor cells or to indirect effect of the tumor. The latter is called Paraneoplastic Neuropathy. Several types have been described. For instance, the method of the present inventions can be used to manage sensory neuropathy associated with lung cancer. Likewise, the method of the present invention can be used to treat neuropathies associated with multiple myeloma. In some embodiments, the method of the present invention is suitable for the treatment of neuropathies associated with Waldenstrom's Macroglobulemia, Chronic Lymphocytic Leukemia, or B-cell Lymphoma. In some embodiments, the method of the present invention is used as part of therapeutic protocol for the treatment of patients with cancers where neuropathy is a consequence of local irradiation or be caused by a chemotherapeutic agent. Chemotherapeutic agents known to cause sensory and/or motor neuropathies include vincristine, an antineoplastic drug used to treat haematological malignancies and sarcomas, as well as cisplatin, taxol and others. The neurotoxicity is dose-related, and exhibits as reduced intestinal motility and peripheral neuropathy, especially in the distal muscles of the hands and feet, postural hypotension, and atony of the urinary bladder. Similar problems have been documented with taxol and cisplatin (MoUman, J. E., 1990, New Eng Jour Med. 322:126-127), although cisplatin-related neurotoxicity can be alleviated with nerve growth factor (NGF) (Apfel, S. C. et al, 1992, Annals of Neurology 31 :76-80). Although the neurotoxicity is sometimes reversible after removal of the neuro toxic agent, recovery can be a very slow process (Legha, S., 1986, Medical Toxicology 1 :421-427; Olesen, et al, 1991, Drug Safety 6:302-314). In some embodiments, the method of the present invention is particularly suitable for the treatment of peripheral neuropathies induced by...
inhibitor of the proteasome such as bortezomib. Bortezomib, chemical name: \[ (\text{R}) -3 - \text{methyl}-1_ - [(2S) -1 - \text{oxo-3} - \text{phenyl-2}_ - [(\text{pyrazin-carboxy}) \text{ amino}] \text{ propyl}] \text{ amino } ] \text{ butyl} \\
boronic acid, was the first to enter the clinical application of proteasome inhibitor, is currently approved by the FDA recommended for multiple myeloma (MM) and mantle cell lymphoma.

In some embodiments, the method of the present invention is suitable for the treatment of neuropathies caused by infections. Peripheral neuropathies can be caused by infection of the peripheral nerves. Viruses that cause peripheral neuropathies include the AIDS virus, HIV-I, which causes slowly progressive sensory neuropathy, Cytomegalovirus which causes a rapidly progressive paralytic neuropathy, Herpes Zoster which cause Shingles, and Poliovirus which causes a motor neuropathy. Hepatitis B or C infections are sometimes associated with vasculitic neuropathy. Bacterial infections that cause neuropathy include Leprosy which causes a patchy sensory neuropathy, and Diphtheria which can cause a rapidly progressive paralytic neuropathy. Other infectious diseases that cause neuropathy include Lyme disease which is caused by a spirochete, and Trypanosomiasis which is caused by a parasite. Both commonly present with a multifocal neuropathy.

In some embodiments, the method of the present invention is suitable for the treatment of neuropathies caused by nutritional imbalance. Deficiencies of Vitamins B12, B1 (thiamine), B6 (pyridoxine), or E, for example, can produce polyneuropathies with degeneration of peripheral nerve axons. This can be due to poor diet, or inability to absorb the nutrients from the stomach or gut. Moreover megadoses of Vitamin B6 can also cause a peripheral neuropathy, and the method of the present invention can be used as part of a de-toxification program in such cases.

In some embodiments, the method of the present invention is suitable for the treatment of neuropathies arising in kidney diseases. Chronic renal failure can cause a predominantly sensory peripheral neuropathy with degeneration of peripheral nerve axons.

In some embodiments, the method of the present invention is suitable for the treatment of hypothyroid neuropathies. Hypothyroidism is sometimes associated with a painful sensory polyneuropathy with axonal degeneration. Mononeuropathy or Mononeuropathy Multiplex can also occur due to compression of the peripheral nerves by swollen tissues.
In some embodiments, the method of the present invention is suitable for the treatment of neuropathies caused by Alcohol and Toxins. Certain toxins can cause Peripheral Neuropathy. Lead toxicity is associated with a motor neuropathy; arsenic or mercury cause a sensory neuropathy, Thalium can cause a sensory and autonomic neuropathy, several of the organic solvents and insecticides can also cause polyneuropathy. Alcohol is directly toxic to nerves and alcohol abuse is a major cause of neuropathy. The method of the present invention can be used, in some embodiments, as part of a broader detoxification program. In still another embodiment, the method of the present invention can be used for the treatment of neuropathies caused by drugs. Several drugs are known to cause neuropathy. They include, among others, nitrofurantoin, which is used in pyelonephritis, amiodarone in cardiac arrhythmias, disulfiram in alcoholism, ddC and ddl in AIDS, and dapsone which is used to treat Leprosy. As above, the method of the present invention can be used, in some embodiments, as part of a broader detoxification program.

In some embodiments, the method of the present invention is suitable for the treatment of neuropathies caused by trauma or compression. Localized neuropathies can result from compression of nerves by external pressure or overlying tendons and other tissues. The best known of these are the Carpal Tunnel Syndrome which results from compression at the wrist, and cervical or lumbar radiculopathies (Sciatica) which result from compression of nerve roots as they exit the spine. Other common areas of nerve compression include the elbows, armpits, and the back of the knees. The method of the present invention is also useful in variety of idiopathic neuropathies. The term "idiopathic" is used whenever the cause of the neuropathy cannot be found. In these cases, the neuropathy is classified according to its manifestations, i.e., sensory, motor, or sensorimotor idiopathic polyneuropathy.

As used herein the term "Crb3-HPO-YAP signaling pathway" refers to the signaling pathway which leads after the activation of Crb3 to the sequestration of YAP in the cytoplasm of a Schwann cell. The main components of the Crb3-HPO-YAP signaling pathway include Crb3, Merlin Willin, Mstl/2 and Latsl/2 and YAP. In particular, the term "YAP" has its general meaning in the art and refers to the Yes-associated protein (YAP) transcription co-activator. Functionally, YAP is a transcription co-activator and a major downstream effector of the Hippo-YAP pathway (Dong et al, 2007). Latsl/2 inhibit YAP by direct phosphorylation, which results in YAP cytoplasmic sequestration (Dong et al, 2007; Hao et al, 2008; Zhao et al, 2007). The unphosphorylated YAP localizes in the nucleus and acts
mainly through TEAD family transcription factors to stimulate expression of genes that promote proliferation and inhibit apoptosis (Zhao et al., 2008). Phosphorylation of YAP by Lats1/2 kinases can also promote its ubiquitination-dependent degradation (Zhao et al, 2010b). As used herein the term "Crb3" has its general meaning in the art ant refers to crumbs family member 3. This protein was originally described as playing a role in epithelial cell polarity and is associated with tight junctions at the apical surface of epithelial cells. Accordingly the term "inhibitor of the Crb3-HPO-YAP signaling pathway" refers to any compound natural or not which have the ability to inhibit said signaling pathway and which leads to the activation of YAP by promoting its nuclear localization.

In some embodiments, the inhibitor of the Crb3-HPO-YAP signaling pathway is an antibody. In some embodiments, the inhibitor of the Crb3-HPO-YAP signaling pathway is anti-Crb3 antibody. The term "antibody" is thus used to refer to any antibody-like molecule that has an antigen binding region, and this term includes antibody fragments that comprise an antigen binding domain such as Fab', Fab, F(ab')2, single domain antibodies (DABs), TandAbs dimer, Fv, scFv (single chain Fv), dsFv, ds-scFv, Fd, linear antibodies, minibodies, diabodies, bispecific antibody fragments, bibody, tribody (scFv-Fab fusions, bispecific or trispecific, respectively); sc-diabody; kappa(lamda) bodies (scFv-CL fusions); BiTE (Bispecific T-cell Engager, scFv-scFv tandems to attract T cells); DVD-Ig (dual variable domain antibody, bispecific format); SIP (small immunoprotein, a kind of minibody); SMIP ("small modular immunopharmaceutical" scFv-Fc dimer; DART (ds-stabilized diabody "Dual Affinity ReTargeting"); small antibody mimetics comprising one or more CDRs and the like. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art (see Kabat et al, 1991, specifically incorporated herein by reference). Diabodies, in particular, are further described in EP 404, 097 and WO 93/1 161; whereas linear antibodies are further described in Zapata et al. (1995). Antibodies can be fragmented using conventional techniques. For example, F(ab')2 fragments can be generated by treating the antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments. Papain digestion can lead to the formation of Fab fragments. Fab, Fab' and F(ab')2, scFv, Fv, dsFv, Fd, dAbs, TandAbs, ds-scFv, dimers, minibodies, diabodies, bispecific antibody fragments and other fragments can also be synthesized by recombinant techniques or can be chemically synthesized. Techniques for producing antibody fragments are well known and described in the art. For example, each of Beckman et al, 2006; Holliger & Hudson, 2005; Le Gall et al, 2004; Reff & Heard, 2001 ;
Reiter et al, 1996; and Young et al, 1995 further describe and enable the production of effective antibody fragments.

In one embodiment of the antibodies or portions thereof described herein, the antibody is a monoclonal antibody. In one embodiment of the antibodies or portions thereof described herein, the antibody is a polyclonal antibody. In one embodiment of the antibodies or portions thereof described herein, the antibody is a humanized antibody. In one embodiment of the antibodies or portions thereof described herein, the antibody is a chimeric antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a light chain of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a heavy chain of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a Fab portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a F(ab')2 portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a Fc portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a Fv portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a variable domain of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises one or more CDR domains of the antibody.

Monoclonal antibodies may be generated using the method of Kohler and Milstein (Nature, 256:495, 1975). To prepare monoclonal antibodies useful in the invention, a mouse or other appropriate host animal is immunized at suitable intervals (e.g., twice-weekly, weekly, twice-monthly or monthly) with antigenic forms of Crb3. The animal may be administered a final "boost" of antigen within one week of sacrifice. It is often desirable to use an immunologic adjuvant during immunization. Suitable immunologic adjuvants include Freund's complete adjuvant, Freund's incomplete adjuvant, alum, Ribi adjuvant, Hunter's Titermax, saponin adjuvants such as QS21 or Quil A, or CpG-containing immunostimulatory oligonucleotides. Other suitable adjuvants are well-known in the field. The animals may be immunized by subcutaneous, intraperitoneal, intramuscular, intravenous, intranasal or other routes. A given animal may be immunized with multiple forms of the antigen by multiple routes. Briefly, the recombinant Crb3 may be provided by expression with recombinant cell
lines. Crb3 may be provided in the form of human cells expressing Crb3 at their surface. Recombinant forms of Crb3 may be provided using any previously described method. Following the immunization regimen, lymphocytes are isolated from the spleen, lymph node or other organ of the animal and fused with a suitable myeloma cell line using an agent such as polyethylene glycol to form a hybridoma. Following fusion, cells are placed in media permissive for growth of hybridomas but not the fusion partners using standard methods, as described (Coding, Monoclonal Antibodies: Principles and Practice: Production and Application of Monoclonal Antibodies in Cell Biology, Biochemistry and Immunology, 3rd edition, Academic Press, New York, 1996). Following culture of the hybridomas, cell supernatants are analyzed for the presence of antibodies of the desired specificity, i.e., that selectively bind the antigen. Suitable analytical techniques include ELISA, flow cytometry, immunoprecipitation, and western blotting. Other screening techniques are well-known in the field. Preferred techniques are those that confirm binding of antibodies to conformationally intact, natively folded antigen, such as non-denaturing ELISA, flow cytometry, and immunoprecipitation.

Significantly, as is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W. R. (1986) The Experimental Foundations of Modern Immunology Wiley & Sons, Inc., New York; Roitt, I. (1991) Essential Immunology, 7th Ed., Blackwell Scientific Publications, Oxford). The Fc′ and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc′ region has been enzymatically cleaved, or which has been produced without the pFc′ region, designated an F(ab′)2 fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of
the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3).

The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity. It is now well-established in the art that the non CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. This invention provides in certain embodiments compositions and methods that include humanized forms of antibodies. As used herein, "humanized" describes antibodies wherein some, most or all of the amino acids outside the CDR regions are replaced with corresponding amino acids derived from human immunoglobulin molecules. Methods of humanization include, but are not limited to, those described in U.S. Pat. Nos. 4,816,567, 5,225,539, 5,585,089, 5,693,761, 5,693,762 and 5,859,205, which are hereby incorporated by reference. The above U.S. Pat. Nos. 5,585,089 and 5,693,761, and WO 90/07861 also propose four possible criteria which may used in designing the humanized antibodies. The first proposal was that for an acceptor, use a framework from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized, or use a consensus framework from many human antibodies. The second proposal was that if an amino acid in the framework of the human immunoglobulin is unusual and the donor amino acid at that position is typical for human sequences, then the donor amino acid rather than the acceptor may be selected. The third proposal was that in the positions immediately adjacent to the 3 CDRs in the humanized immunoglobulin chain, the donor amino acid rather than the acceptor amino acid may be selected. The fourth proposal was to use the donor amino acid reside at the framework positions at which the amino acid is predicted to have a side chain atom within 3A of the CDRs in a three dimensional model of the antibody and is predicted to be capable of interacting with the CDRs. The above methods are merely illustrative of some of the methods that one skilled in the art could employ to make humanized antibodies. One of ordinary skill in the art will be familiar with other methods for antibody humanization. In one embodiment of the humanized forms of the antibodies, some, most or all of the amino acids outside the CDR regions have been replaced with amino acids from human immunoglobulin molecules.
but where some, most or all amino acids within one or more CDR regions are unchanged. Small additions, deletions, insertions, substitutions or modifications of amino acids are permissible as long as they would not abrogate the ability of the antibody to bind a given antigen. Suitable human immunoglobulin molecules would include IgGl, IgG2, IgG3, IgG4, IgA and IgM molecules. A "humanized" antibody retains a similar antigenic specificity as the original antibody. However, using certain methods of humanization, the affinity and/or specificity of binding of the antibody may be increased using methods of "directed evolution", as described by Wu et al, /Mol. Biol. 294:151, 1999, the contents of which are incorporated herein by reference.

Fully human monoclonal antibodies also can be prepared by immunizing mice transgenic for large portions of human immunoglobulin heavy and light chain loci. See, e.g., U.S. Pat. Nos. 5,591,669, 5,598,369, 5,545,806, 5,545,807, 6,150,584, and references cited therein, the contents of which are incorporated herein by reference. These animals have been genetically modified such that there is a functional deletion in the production of endogenous (e.g., murine) antibodies. The animals are further modified to contain all or a portion of the human germ-line immunoglobulin gene locus such that immunization of these animals will result in the production of fully human antibodies to the antigen of interest. Following immunization of these mice (e.g., XenoMouse (Abgenix), HuMAb mice (Medarex/GenPharm)), monoclonal antibodies can be prepared according to standard hybridoma technology. These monoclonal antibodies will have human immunoglobulin amino acid sequences and therefore will not provoke human anti-mouse antibody (KAMA) responses when administered to humans. In vitro methods also exist for producing human antibodies. These include phage display technology (U.S. Pat. Nos. 5,565,332 and 5,573,905) and in vitro stimulation of human B cells (U.S. Pat. Nos. 5,229,275 and 5,567,610). The contents of these patents are incorporated herein by reference.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab') 2 Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric F(ab')2 fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by
homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDR1 and/or CDR2 regions have been replaced by homologous human or non-human sequences. The present invention also includes so-called single chain antibodies.

The various antibody molecules and fragments may derive from any of the commonly known immunoglobulin classes, including but not limited to IgA, secretory IgA, IgE, IgG and IgM. IgG subclasses are also well known to those in the art and include but are not limited to human IgGl, IgG2, IgG3 and IgG4.

In another embodiment, the antibody according to the invention is a single domain antibody. The term "single domain antibody" (sdAb) or "VHH" refers to the single heavy chain variable domain of antibodies of the type that can be found in Cameld mammals which are naturally devoid of light chains. Such VHH are also called "nanobody®". According to the invention, sdAb can particularly be llama sdAb.

In some embodiments, the inhibitor of the Crb3-HPO-YAP signaling pathway is an aptamer directed against Crb3. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by Exponential enrichment (SELEX) of a random sequence library. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence.

In some embodiments, the inhibitor of the Crb3-HPO-YAP signaling pathway is an inhibitor of expression of one component of said pathway which leads to the activation of YAP in the Schwann cell. In some embodiments, the inhibitor of expression is selected from the group consisting of inhibitors of Crb3 expression, inhibitor of Merlin expression, inhibitors of Willin expression, inhibitors of Mstl/2 expression and inhibitors of Latsl/2 expression.

An "inhibitor of expression" refers to a natural or synthetic compound that has a biological effect to inhibit the expression of a gene. For example, an "inhibitor of expression"
denotes a natural or synthetic compound that has a biological effect to inhibit the expression of the targeted gene.

Inhibitors of gene expression for use in the present invention may be based on antisense oligonucleotide constructs. Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of the targeted mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of the targeted protein, and thus activity, in a cell. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding the target protein can be synthesized, e.g., by conventional phosphodiester techniques and administered by e.g., intravenous injection or infusion. Methods for using antisense techniques for specifically inhibiting gene expression of genes whose sequence is known are well known in the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732).

Small inhibitory RNAs (siRNAs) can also function as inhibitors of gene expression for use in the present invention. Gene expression can be reduced by contacting the tumor, subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that gene expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschi, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836).

Ribozymes can also function as inhibitors of gene expression for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of the targeted mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target
molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays.

Both antisense oligonucleotides and ribozymes useful as inhibitors of gene expression can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramidite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

Antisense oligonucleotides siRNAs and ribozymes of the invention may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide siRNA or ribozyme nucleic acid to the cells. Preferably, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the the antisense oligonucleotide siRNA or ribozyme nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known
to the art. Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in KRIEGLER (A Laboratory Manual," W.H. Freeman CO., New York, 1990) and in MURRY ("Methods in Molecular Biology," vol.7, Humana Press, Inc., Clifton, N.J., 1991). Preferred viruses for certain applications are the adeno-viruses and adeno-associated viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hematopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g., SANBROOK et al, "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly advantageous for this because they do not have the same safety concerns as with many of the
viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUC19, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

In some embodiments, the inhibitor of the Crb3-HPO-YAP signaling pathway is administered to the subject in combination with a further therapeutic agent. In some embodiments, the inhibitor of the Crb3-HPO-YAP signaling pathway is administered in combination with a neuregulin polypeptide. By "neuregulin" or "NRG" is meant a polypeptide that is encoded by an NRG-1, NRG-2, or NRG-3 gene or nucleic acid (e.g., a cDNA), and binds to and activates ErbB2, ErbB3, or ErbB4 receptors, or combinations thereof. By "neuregulin-1," "NRG-1," "heregulin," "GGF2," or "p185erbB2 ligand" is meant a polypeptide that binds directly to or transactivates the ErbB2 receptor and is encoded by the pl85erbB2 ligand gene described in U.S. Pat. No. 5,530,109; U.S. Pat. No. 5,716,930; and U.S. Pat. No. 7,037,888, the contents of each of which are incorporated herein by reference. Polypeptides encoded by the NRG-1, NRG-2, and NRG-3 genes possess EGF-like domains that allow them to bind to and activate ErbB receptors. Holmes et al. (Science 256: 1205-1210, 1992) have shown that the EGF-like domain alone is sufficient to bind and activate the pl85erbB2 receptor. Accordingly, any polypeptide product encoded by the NRG-1, NRG-2, or NRG-3 gene, e.g., a polypeptide having an EGF-like domain encoded by a neuregulin gene or cDNA (e.g., an EGF-like domain, as described in U.S. Pat. No. 5,530,109; U.S. Pat. No. 5,716,930; U.S. Pat. No. 7,037,888, U.S. Pat. No. 7,135,456, and U.S. Pat. No. 7,319,019; or an EGF-like domain as disclosed in WO 97/09425) may be used in the methods of the invention. In some embodiments, the inhibitor of the Crb3-HPO-YAP signaling pathway is administered in combination with an activator of TACE (gamma-secretase) such as NIASPAN® (niacin extended-release tablets) which are available from Kos.
As used herein, the term "effective amount" refers to a quantity sufficient to achieve a therapeutic effect (e.g. myelin elongation). In the context of therapeutic or prophylactic applications, the amount of a composition administered to the subject will depend on the type and severity of the disease and on the characteristics of the individual, such as general health, age, sex, body weight and tolerance to drugs. It will also depend on the degree, severity and type of disease. The skilled artisan will be able to determine appropriate dosages depending on these and other factors. In some embodiments, an effective amount of the inhibitor of the Crb3-HPO-YAP signaling pathway for achieving a therapeutic or prophylactic effect, range from about 0.000001 mg per kilogram body weight per day to about 10,000 mg per kilogram body weight per day. Typically, the dosage ranges are from about 0.0001 mg per kilogram body weight per day to about 100 mg per kilogram body weight per day. For example dosages can be 1 mg/kg body weight or 10 mg/kg body weight every day, every two days or every three days or within the range of 1-10 mg/kg every week, every two weeks or every three weeks. In some embodiments, a single dosage of peptide ranges from 0.1-10,000 micrograms per kg body weight. In some embodiments, aromatic- cationic peptide concentrations in a carrier range from 0.2 to 2000 micrograms per delivered milliliter.

Typically, the inhibitor of the Crb3-HPO-YAP signaling pathway is combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form pharmaceutical compositions. "Pharmaceutically" or "pharmaceutically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. Typically, the pharmaceutical compositions contain vehicles, which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or
dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. Sterile injectable solutions are prepared by incorporating the Inhibitor of the Crb3-HPO-yap signaling pathway in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

A further aspect of the present invention relates to a nucleic acid molecule encoding for a YAP polypeptide for use in the treatment of a peripheral neuropathy as described above. In some embodiments, the nucleic acid molecule is used with a vector. In some embodiments, the vector is able to transfect the Schwann cells in a selective manner so that the transgene is specifically expressed by the Schwann cells. In some embodiments, the YAP polypeptide is a dominant active form or a dominant negative form of YAP (e.g. YAP-S127A as described in the EXAMPLE).

A further aspect of the present invention relates to a method for screening a drug for the treatment of peripheral neuropathies comprising the steps of i) providing a test compound, ii) determining whether the test compound is able to inhibit the Crb3-HPO-YAP signaling pathway and iii) positively selecting the test compound when it inhibits the Crb3-HPO-YAP signaling pathway.

In some embodiments, the screening method of the invention is performed in a Schwann cell.

In some embodiments, the screening method of the invention comprises the steps of determining whether the test compound is able to bind to Crb3. Any method well known in the art for determining whether the test compound is able to bind to Crb3 may be used.
In some embodiments, the screening method of the invention comprises the step of determining whether the test compound is able to activate YAP. In particular, the screening method of the invention comprises the step of determining whether the test compound is able to promote the nuclear localization of YAP. In some embodiments, the screening method of the invention comprises the step of determining whether the test compound is able to activate the expression of at least one gene for which the expression is controlled by YAP. For instance any YAP dependent transcription assay may be used for determining whether the test compound is able to activate expression of at least one gene for which the expression is controlled by YAP. In some embodiments, a reporter gene is used. In one embodiment, the reporter gene encodes one of the groups consisting of GFP, CAT, GAL, LUC, and GUS. For example, a sensitive cell-based YAP reporter assay, which consists of a luciferase reporter and a TEAD transcription factor may be used. This reporter activity is strongly stimulated by expression of YAP, which binds to and activates TEAD in transcription.

The above assays may be performed using high throughput screening techniques for identifying test compounds for developing drugs that may be useful to the treatment of peripheral neuropathies. High throughput screening techniques may be carried out using multi-well plates (e.g., 96-, 389-, or 1536-well plates), in order to carry out multiple assays using an automated robotic system. Thus, large libraries of test compounds may be assayed in a highly efficient manner. A typical strategy for identifying test compounds starts with cultured cells transfected with a reporter gene fused to the promoter of any gene that is activated by YAP. Compounds in the library will be applied one at a time in an automated fashion to the wells of the microtiter dishes containing the transgenic cells described above. The whole cell assay of the invention described herein can be performed, for example, by utilizing pre-packaged kits comprising any or all of the reagents of the assay, such as a solid phase coated with a binding partner to a protein of interest, or a detection molecule. The cell-free assays of the invention may be performed, for example, by utilizing pre-packaged kits comprising any or all of the reagents of the assay.

In some embodiments, the screening method of the invention comprises a step of performing a functional assay. In some embodiments, an in vitro myelination model as described in the EXAMPLE may be used. For examples the test compound is put in contact with non-elongating myelinating cells in culture and production of myelin is then measured. It is then possible to positively select test compound that are able to produce an increased
amount of myelin (in comparison with a control cells which are not put in contact with the test compound) when e.g. the test compound was initially selected to force YAP activity or to lift Crb3 inhibition. In vivo assays may also be perfomed as described in the EXAMPLE.

In some embodiments, the test compound of may be selected from the group consisting of peptides, peptidomimetics, small organic molecules, antibodies, aptamers or nucleic acids. For example the test compound according to the invention may be selected from a library of compounds previously synthesized, or a library of compounds for which the structure is determined in a database, or from a library of compounds that have been synthesized de novo. In a particular embodiment, the test compound may be selected form small organic molecules. As used herein, the term "small organic molecule" refers to a molecule of size comparable to those organic molecules generally sued in pharmaceuticals. The term excludes biological macromolecules (e.g.; proteins, nucleic acids, etc.); preferred small organic molecules range in size up to 2000 Da, and most preferably up to about 1000 Da.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

EXAMPLE:

Results:

We found that the apical polarity protein Crb3, one of the mammalian homologs of drosophila Crumbs, is expressed in the peripheral nerve and specifically distributes to the microvilli of myelinating Schwann cells (mSCs). These microvilli are fine cellular protrusions that originate from the outer membrane of the Schwann cell, mix with microvilli of neighboring cells and contact the node of Ranvier. Crb3 immunostaining on teased fibers of mouse sciatic nerves appeared as two lines at the extremities of the myelinating Schwann cell where it colocalized with known microvilli marker moesin. Moreover when confocal scans were projected on the z-axis Crb3 staining appeared as a ring that surrounded the node of Ranvier.
To address the role of Crb3 in mSC microvilli we designed lentiviral vectors expressing mouse Crb3 shRNAs or control shRNA under the control of a U6 promoter together with Dsred2 under a CMV promoter. We injected these lentiviruses into sciatic nerves of new born mouse pups before the start of myelination (postnatal days 3 to 5). Under these conditions lentiviral vectors infect myelinating Schwann cells but not neurons. Two months later - when myelination is near completion - these mice were sacrificed and the injected sciatic nerves were fixed. Myelinated fibers were teased on glass slides, immunostained and analyzed using confocal microscopy. As expected infected myelinating Schwann cells expressing Dsred2 and Crb3 shRNA expressed less Crb3 than non-infected cells. We also observed that cells silenced for Crb3 were much longer than control cells, suggesting that Crb3 is an inhibitor of myelination. Indeed on the same axon Crb3-silenced cells were typically longer than non-infected neighbouring cells. However the fiber diameter did not change. To check whether Crb3 silencing affected the myelin sheath ultrastructure we replaced Dsred2 with Placental Alkaline Phosphatase (PLAP) in our lentiviral vectors. Using the fine black precipitate indicative of PLAP enzymatic activity, we detected infected myelinating Schwann cells using electron microscopy. We found no particular defect in the ultrastructure of Crb3-silenced cells, suggesting that myelin was correctly formed. The g-ratio (axon diameter/fiber diameter) of infected cells was not different from non-infected surrounding cells, confirming that Crb3 silencing did not significantly affect myelin thickness in vivo. Moreover the myelin interperiodic distance of Crb3-silenced cells was not different from non-infected cells showing that the compact myelin was normal. We observed labeled nodes of Ranvier where the microvilli contacted the nodal membrane and did not appear disorganized. Microvilli and node of Ranvier markers were also correctly expressed and localized, suggesting that the reduction of Crb3 expression did not affect microvilli or adjacent nodes of Ranvier. Finally we overexpressed Crb3 or control GFP in the mouse sciatic nerve. The majority of Crb3 over-expressing cells did not display a myelinating phenotype confirming that Crb3 inhibited myelination. Taken together these data indicate that Crb3 inhibits myelination by limiting myelin sheath elongation in vivo.

Crb3 has been suggested to activate the HPO pathway, via Merlin and/or Willin (also termed FRMD6), to inhibit the function of YAP (Yes Associated Protein) transcription cofactor and its paralog TAZ (Transcriptional Co-activator with PDZ binding motifs) to control cell proliferation, cell death and tissue growth. While Merlin is known to be expressed in Schwann cells, we also detected the major components of the HPO pathway Willin, Mstl/2
and Latsl/2, and YAP in the myelinated mouse peripheral nerve. In immunostaining Willin localized to the Schwann cell microvilli while Merlin localized in Cajal's bands. In contrast YAP was mostly nuclear. Endogenous levels of Mstl/2 and Latsl/2 were too low to be detected by immunohistochemistry. These localizations suggested that Willin is the target of Crb3 and subsequent activation of the HPO pathway could inhibit YAP with consequences for Schwann cell growth. We therefore silenced Willin in Schwann cells \textit{in vivo} using the viral approach described previously. Willin silencing increased the myelin internodal length at two months compared to control shRNA, showing that Willin and the HPO pathway participates in the control of myelin elongation. We then examined the consequence of silencing the final target of this pathway, YAP. Without YAP, Schwann cells remained shorter than controls and were also significantly thinner (6.2±0.27 vs 7.4±0.27 µm; P<0.01), showing that YAP is required for Schwann cell myelination \textit{in vivo}.

To determine if Crb3 indeed controls YAP activity, we measured the amount of nuclear YAP in Crb3-silenced cells and found that the lack of Crb3 promotes YAP nuclear localization. In addition, using co-infection with two viruses, we reduced YAP expression in the over-elongating Crb3-silenced cells. In this condition mSCs over-elongated but significantly less than when Crb3 alone was silenced, showing that Crb3 requires YAP to mediate its effect on myelination. We conclude that Crb3, in the microvilli, controls myelin sheath elongation via the HPO pathway that negatively regulates YAP.

YAP is a transcription cofactor that is known to regulate gene expression through its binding to transcription factors including the TEAD factors. We therefore investigated whether YAP could control the transcription of myelin genes, such as Myelin Protein Zero (MPZ), the main peripheral myelin protein, and KroX20, a key myelin gene regulator that controls MPZ expression. Cells expressing MPZ or KroX20 promoters driving the expression of luciferase were transfected with control GFP or different YAP and TEAD constructs and the resulting luciferase expression was measured. We found that YAP stimulated the KroX20 promoter but not the MPZ promoter. When cells co-expressed YAP and TEAD1, stimulation of the KroX20 and MPZ promoters were maximal. Co-expressing YAP with TEAD4 stimulated the KroX20 promoter with lower efficiency but not MPZ, suggesting that the effects of YAP on MPZ transcription may occur through KroX20 expression. Taken together these data indicate that YAP strongly promotes KroX20 transcription via its interaction with TEAD 1 corroborating the role of YAP during myelination.
We also used an *in vitro* myelination model to analyze the roles of Crb3 and YAP. Mouse embryonic dorsal root ganglia were dissociated and seeded on Matrigel coated coverslips. Proliferating Schwann cells were then infected with retroviruses expressing Crb3 or control shRNAs. After induction of myelination, we found that Crb3 silencing reduced the number of myelin segments formed but did not affect their lengths. This suggested that the elongation mechanism regulated by Crb3 is not functional *in vitro*. Indeed the average Schwann cell length in co-cultures decreased following induction of myelination (192±1.8 vs 164±2.3 μm; P < 0.001), indicating that myelin segments do not elongate *in vitro*. We therefore tried to force myelin elongation by expressing a dominant active form of YAP, YAP-S127A, in cultured cells. Under these conditions the myelin sheath did not get longer and was instead thicker. Moreover some myelinating cells displayed an abnormal "bubbling" myelin sheath and started to demyelinate. These effects were amplified when Crb3 was silenced in addition, confirming that Crb3 inhibits YAP activity *in vitro* too. Taken together these data indicated that non-elongating myelinating cells in culture produce more myelin when both YAP activity is forced and Crb3 inhibition is lifted. As this myelin cannot be deposited longitudinally it accumulates radially around the axon leading to a thicker myelin sheath and, in excess, to abnormal myelin "bubbling". Hypermyelination increases myelin instability leading to demyelination.

Our attempts to increase YAP activity in mSCs by overexpressing dominant active YAP-S127A lead to strong myelin degeneration. In order to control the expression level of the protein better we used a transgenic mouse line that expresses YAP-S127A under the control of an inducible TetOn promoter and we transduced cells with a virus expressing the tet-dependent transactivator (tTA). Again demyelination was induced in YAP-S127A expressing cells within 15 days. However just nine days after inducing YAP-S127A expression, before they demyelinate, transgenic cells were significantly thicker than control cells, suggesting that YAP overactivity leads to hypermyelination before degeneration *in vivo*.

Obtaining a regular myelin sheath length along a single axon is critical for nerve conduction velocity. However it is not straightforward because the rate of myelin sheath elongation is not uniform during peripheral nerve myelination, starting strong in pups and slowing when mice age. We investigated whether Crb3 and YAP functions could participate in this developmental process. Firstly, the effect of Crb3 silencing was analyzed at different
ages: at 15 days postnatal (dPN) - 10 to 12 days after viral injection - no change could be detected versus control shRNA; at 30 dPN the internodal length had almost doubled in Crb3-silenced cells, with a growth rate more than ten times higher than in control cells (13.1 vs 1.1 µm/day); at 60 dPN the internodal length of Crb3-silenced cells had increased again, but the growth rate decreased to only double of control cells (5.7 vs 2.8 µm/day). These data indicate that Crb3 silencing increases myelin elongation by boosting the growth rate between 15 dPN and 30 dPN without altering the time-frame of the elongation. Moreover when Crb3 was silenced in 30 dPN mice, the loss of Crb3 had only a very mild effect on the internodal length. As Crb3 is still significantly expressed in adult mouse nerve this suggests that Crb3 loses its function at adult ages. Consistently the expression of Crb3 target, YAP, in the nucleus of mSCs decreased between 5 dPN and 60 dPN, suggesting that nuclear YAP is regulated during peripheral nerve myelination and constitutes a limiting factor for both myelin elongation and Crb3 function.

In vivo experiments suggested that active nuclear YAP is required for myelin sheath elongation but it is not sufficient to elongate the sheath in cultures in vitro. To explain this discrepancy we hypothesized that the axonal elongation, which occurs with body growth in vivo but not in cultures, is required to drive myelin elongation through nuclear YAP expression. To test this hypothesis we used a femoral distraction technique to mechanically stretch axons in the sciatic nerve of adult mice. This technique enabled the extension of the sciatic nerve by ~ 30% over 15 days in living mice. As previously observed in rats, axonal stretching in adults steadily stimulated myelin sheath elongation. This correlated with increased YAP expression in the nucleus of myelinating cells. When cells were silenced for YAP they still elongated but this elongation correlated with a demyelinating phenotype, suggesting that YAP-silenced cells could not 'keep up' with the imposed elongation. When Crb3 was silenced in mSCs before extending the adult nerve, cells produced a myelin sheath even longer than control infected cells in similarly extended nerves. Accordingly nuclear YAP levels also increased even more in the absence of Crb3, indicating that the function of Crb3 can be re-engaged in adult nerves by axonal stretching through YAP nuclear activity. The myelin elongation mechanism is therefore initiated by an axonal stretch that recruits YAP to the nucleus of Schwann cells; it is then negatively controlled by Crb3, via the HPO pathway, to allow the harmonious growth of all myelin segments along the axon.
Reduced internodal length strongly impairs nerve conduction velocity and some human peripheral neuropathies have been shown to be linked to reduced internodal length such as CMT4F (Periaxin gene mutations), and congenital muscular dystrophy 1A (α2 laminin gene mutations). To check whether the Crb3/HPO/YAP mechanism is involved in these diseases we investigated a naturally-occurring mouse line, Dy21, mutated on α2 laminin gene that displays both a peripheral neuropathy and a reduced internodal length. We found that mSCs of mutant mice had significantly less nuclear YAP than control littermates, suggesting that YAP deficiency may be responsible for the reduced internodal length. To confirm this we attempted to express dominant active YAP-S127A in mutant Schwann cells but this was deleterious for the mice. So in order to increase the residual YAP nuclear activity we silenced Crb3 in mutant cells. This indeed increased the amount of nuclear YAP and accordingly increased the internodal length of mutant cells, showing that the lack of nuclear YAP underlies the peripheral nerve defect in these mice. Our data therefore suggests that peripheral neuropathies with reduced internodal length are due to defects in the expression/activation of YAP in the Schwann cell nucleus and it may be possible to correct internodal lengths by artificially promoting YAP activity via manipulation of Crb3.

**Discussion:**

Our data show that YAP activity is essential for the stimulation of peripheral nerve myelination in vivo and in vitro. We propose that this pathway complements the Nrgl-ErbB-PI3K-AKT pathway. Indeed the manipulation of this Nrgl-initiated pathway revealed that other mechanisms were likely to be involved in the promotion of Schwann cell myelination and in oligodendrocytes, the myelinating glia of the central nervous system. The YAP pathway may represent one of these supplementary mechanisms.

In addition to being complementary, it is likely that both the Nrgl and the YAP pathway interact in mSCs. Indeed, while altering the Nrgl pathway affects the myelin thickness and not the myelin length, the manipulation of the YAP pathway can lead to changes in both parameters depending on whether the myelin can be deposited longitudinally or not. Typically in myelinating co-cultures no myelin elongation occurs because axons do not elongate. Therefore the expression of a dominant active form of YAP the resulting overmyelination accumulates radially leading to thicker myelin and abnormal myelin "bubbling". A similar event happens in vivo in the inducible dominant active YAP mice: the
expression of the same constitutively active protein forces cells to produce more myelin than
natural nerve elongation requires so cells accumulate excess myelin radially and get thicker.
Mechanistically it has been reported that YAP can be phosphorylated by AKT and that YAP
functionally interacts with mTOR signaling, thus interconnections between the Nrgl and the
YAP pathways may occur at this level.

It nevertheless remains unclear why Crb3-silenced cells "choose" to elongate their
myelin sheath instead of making it thicker. Under the same developmental conditions, Dlgl-
silenced cells make thicker rather than longer myelin by inhibition of the Nrgl-ErbB-PI3K-
AKT pathway. This suggests that beyond the production of myelin and the potential
interaction between YAP and Nrgl pathways, Crb3 is involved in a mechanism that not only
inhibits myelination but also constrains the longitudinal deposition of myelin. It would be
interesting to examine the consequence of relieving Crb3 inhibition while increasing
myelination via the Nrgl-ErbB-PI3K-AKT pathway. In this case the excess of myelin might
be deposited longitudinally instead of making the myelin thicker.

Our data suggests that Crb3 could act as a sensor in the microvilli for signals coming
from neighboring cells or from the axonal node of Ranvier. Activated by these signals Crb3
could activate the HPO pathway, phosphorylate and prevent YAP access to the nucleus and
thereby inhibit myelination. Although Crb3 has a short extracellular domain it remains
unclear whether it can interact with the extracellular matrix or with other proteins
extracellular domains in cis or in trans. Another possibility could be that Crb3 interacts with
its transmembrane domain or its intracellular domain with other transmembrane proteins
that will act as the sensor protein for extracellular signals. Willin was first detected in peripheral
nerve tissues as an interacting protein for Neurofascin using two-hybrid technique (Gunn-
Moore) and Neurofascin 155 (NF155) is expressed in some conditions in the SC microvilli;
so it is possible that Crb3 interacts with Willin and NF155 in mSC microvilli. NF155 may
therefore be the transmembrane protein responsible for sensing the extracellular signals and
for translating it to mSC via Crb3 and the HPO pathway. However no data indicates that the
loss or the inactivation of NF155 in mSCs results in longer internodal length as it could be
expected if you block the inhibitory Crb3/HPO pathway. Additional work will be required to
discover the mechanism by which Crb3 activity is engaged during the control of myelination
in peripheral nerves.
A major implication of our data is that nerve extension promotes nuclear YAP localization and stimulates myelin sheath elongation. These data are consistent with empirical observations showing that myelin internodal length correlates with the limb growth during development. Nevertheless, the molecular mechanisms that underlie this process remain unclear. When nerves are extended axons are stretched and they elongate by inserting new material all along their length. This elongation mechanism is different from the axonal growth that occurs during embryonic development and in vitro, which is mediated by the axonal tip. Our data suggest that the extending axons send a signal to the mSC. This signal might originate from axo-glial junctions that link glial paranodal loops to paranodes of the node of Ranvier. As it is lying directly on the axon, the Schwann cell can detect axonal stretching if both glial extremities are anchored on the stretched axon and the glial cytoskeleton serves as a tension sensor.

The physical interactions between the glial abaxonal (opposite of the axon) plasma membrane, its underlying cytoskeleton and the extracellular matrix could also play a critical role in the detection of axonal stretching. The abaxonal membrane contains dystroglycans that interact with the laminin of the basal lamina. Inside the cell these dystroglycans interact with Periaxin or Utrophin, which are linked to the cytoskeleton. Mutations in Periaxin gene lead to Charcot-Marie-Tooth disease 4F, a peripheral neuropathy with reduced internodal length, as observed in congenital dystrophy patients, which are mutated in Laminin-2 gene. In addition mouse mutants for dystroglycans and Utrophin display a reduced internodal length, showing that the interaction between the basal lamina and the glial cytoskeleton is required for myelin elongation. We therefore suggest that the anchoring of the Schwann cell to the basal lamina via dystroglycans and the related cytoskeleton participates in the detection/sensing of axonal stretching by offering a stable reference to oppose to the extending axon.

REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.
CLAIMS:

1. A method of enhancing myelination in a subject in need thereof comprising administering the subject with a therapeutically effective amount of an inhibitor of the Crb3-HPO-YAP signaling pathway.

5 2. The method of claim 1 for correcting myelination and myelin geometry.

3. The method of claim 1 for enhancing and promoting myelin elongation.

4. The method of claim 1 for the treatment of peripheral neuropathies.

5. The method of claim 1 for the treatment of hereditary peripheral neuropathies.


7. The method of claim 1 for the treatment of acquired neuropathies.

8. The method of claim 7 for the treatment of acquired neuropathy selected form the group consisting of diabetic neuropathies, immune-mediated neuropathies, neuropathies associated with vasculitis or inflammation of the blood vessels in peripheral nerves, neuropathies associated with monoclonal gammopathies, neuropathies associated with tumors or neoplasms, neuropathies caused by a chemotherapeutic agent, neuropathies caused by infections, neuropathies caused by nutritional imbalance, hypothyroid neuropathies, neuropathies caused by alcohol and toxins and neuropathies caused by trauma or compression.

9. The method of claim 1 wherein the inhibitor of the Crb3-HPO-YAP signaling pathway is an antibody, such as an anti-Crb3 antibody.

10. The method of claim 1 wherein the inhibitor of the Crb3-HPO-YAP signaling pathway is an inhibitor of expression of one component of said pathway which leads to the activation of YAP in the Schwann cells.

11. The method of claim 10 wherein the inhibitor of expression is selected from the group consisting of inhibitors of Crb3 expression, inhibitor of Merlin expression, inhibitors of Willin expression, inhibitors of Mstl/2 expression and inhibitors of Latsl/2 expression.
12. The method of claim 1 wherein the inhibitor of the Crb3-HPO-YAP signaling pathway
is administered to the subject in combination with a further therapeutic agent such as a neuregulin polypeptide.

13. A method for the treatment of a peripheral neuropathy in a subject in need thereof comprising administering the subject with a therapeutically effective amount of a nucleic acid molecule encoding for a YAP polypeptide.

14. The method of claim 13 wherein the YAP polypeptide is a dominant active form or a dominant negative form of YAP.

15. A method for screening a drug for the treatment of peripheral neuropathies comprising the steps of i) providing a test compound, ii) determining whether the test compound is able to inhibit the Crb3-HPO-YAP signaling pathway and iii) positively selecting the test compound when it inhibits the Crb3-HPO-YAP signaling pathway.
INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/056145

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/47 C07K16/18

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Relevant to claim No.</th>
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<tr>
<td>A</td>
<td>MOLEI RINHO S ET AL: &quot;Wildin, an upstream component of the hippo signaling pathway, orchestrates mammalian peripheral nerve fibroblasts&quot;, PLOS ONE, vol. 8, no. 4, 1 April 2013 (2013-04-01), pages 1-16, XP002729593, Online</td>
<td>13,14</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:
  *A* document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search
12 May 2015

Date of mailing of the international search report
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Authorized officer
Page, Michael

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<td>MAKAROVA O ET AL: &quot;Mammalian Crumbs3 is a small transmembrane protein linked to protein associated with Lin-7 (Pal si)&quot;, GENE, ELSEVIER, AMSTERDAM, NL, vol. 302, no. 1-2, 2 January 2003 (2003-01-02), pages 21-29, XP004402152, ISSN: 0378-1119, DOI: 10.1016/S037811902010843 the whole document</td>
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