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Signaling Crosstalk between the Wnt, BMP and Endolysosomal Pathways in Development and Disease

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Signaling Crosstalk between the Wnt, BMP and Endolysosomal Pathways in Development and Disease

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biological Chemistry

by

Diego Ploper

2015
Few processes are as complex as development from a one cell embryo to a multicellular adult with specialized tissues. However, organisms use a surprisingly small number of signaling pathways for such an intricate task. As a result, the integration and coordination of these few pathways must play a central role in development. Throughout evolution, signaling factors may acquire new functions and be recruited to different pathways, generating novel signaling nodes. This thesis describes two of these innovations involving the Wnt signaling pathway. In the first, a secreted Wnt antagonist acquired new functions by also inhibiting an extracellular protease. In the second, a transcription factor involved in organelle biogenesis was brought under regulation
by Wnt signaling through Glycogen Synthase Kinase 3 (GSK3) phosphorylation sites identified during this work.

The Bone Morphogenetic Protein (BMP) and Wnt pathways pattern the early *Xenopus* embryo. At gastrula, dorsal-ventral (DV) polarity is determined by a gradient of BMP signaling, created and reinforced by an extracellular network of interacting proteins secreted by two gastrula centers, the ventral center and the Spemann organizer. Key factors in the maintenance of this gradient are Chordin, a BMP inhibitor, and tolloid, a metalloproteinase that degrades Chordin. Surprisingly, secreted Frizzled Related Protein (sFRP) antagonists of Wnt signaling were found to have adopted novel roles in the BMP pathway. In particular, the sFRP Crescent, while retaining its anti-Wnt properties, was found in this thesis to competitively inhibit tolloids and protect Chordin from proteolytic degradation. Together with Sizzled, its ventrally expressed counterpart, Crescent regulates DV patterning by hindering the proteolytic activity of an extracellular protease and causing an increase in Chordin.

Wnt signaling requires the endolysosomal pathway in order to sequester negative regulators such as GSK3 and Axin1 inside multivesicular bodies (MVBs). Thus, the Wnt and endolysosomal pathways converge at the level of MVBs. Expansion of the MVB compartment, by lysosomal inhibition or mutations in Presenilin (a protein mutated in Alzheimer’s), caused an enhancement of Wnt signaling mediated by increased sequestration of inside MVBs of GSK3, a key negative regulator of Wnt signaling. A similar expansion of the MVB/late endolysosomal compartment is triggered in cells in which the transcriptional regulators of lysosomal biogenesis are dysregulated.

The melanoma oncogene MITF was shown here to drive the biogenesis of endolysosomes, which upon Wnt stimulation lead to increased sequestration of GSK3 and other
components of the β-Catenin destruction complex. In this way MITF, an oncogene amplified in 20% of melanomas, enhanced canonical Wnt signaling. By inhibiting GSK3 phosphodegron-mediated ubiquitination and proteasomal degradation, Wnt signaling stabilizes a plethora of cellular proteins. This study identifies MITF as a transcription factor that is regulated by GSK3 and Wnt through particular phosphorylation sites. This finding deepens knowledge about this lineage-addiction oncogene that determines melanoma phenotypes.

The work presented in this dissertation covers a wide range of experimental systems, from Xenopus embryos to melanoma cell lines, and addresses unexpected crosstalks of the Wnt signaling pathway in development and disease. A secreted Wnt inhibitor, Crescent, evolved a role in the BMP pathway by inhibiting tolloid proteases. This thesis also underscores the importance of the endolysosomal pathway for canonical Wnt signaling. The discovery of novel phosphorylations in the oncogene MITF, and its role as a driver of endolysosomal biogenesis in melanoma, suggests that Wnt might regulate other MiT family members in the same manner. In the final part of this dissertation we present a review on how endolysosomal biogenesis and Wnt may intersect with MiT family oncogenic activity.
The dissertation of Diego Ploper is approved.

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2015
To Paula, Dalia and Ivan
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Chapter 1

Introduction
In this section I describe the molecular pathways involved in the *Xenopus* dorsal-ventral system.
How do embryos of any particular species reliably and consistently develop time after time? This has always been a central question in developmental biology. In 1924 Hilde Mangold, a graduate student of Hans Spemann at Freiburg University, began paving the way towards understanding this complex enigma. Mangold performed the key experiment of transplanting a small region of the amphibian gastrula, the dorsal lip of the blastopore, into the opposite (ventral) region of host embryos using embryos of different pigmentation (Spemann and Mangold, 1924). Once the host embryos healed, they gave rise to perfect Siamese (conjoined) twins. Undoubtedly, this small graft of tissue had very potent inductive effects on the recipient. Spemann named the region the “organizer”, due to the remarkable fact that the transplanted organizer cells themselves only gave rise to notochord, yet were able to induce neighboring host cells to differentiate into dorsal rather than ventral tissues. In essence, Spemann’s organizer was able to instruct adjacent cells to follow a particular differentiation route. In 1935, Hans Spemann received the Nobel Prize for Physiology or Medicine for this research on the embryonic induction by organizer tissue.

Amphibian embryos provide an extremely amenable system for tackling questions in vertebrate embryogenesis and biomedical research. Since they develop ex utero (in a pond or, in our case, in a dish), their development can be easily followed. Their large size (approximately 1.2 mm in diameter) and fast rate of development (from one cell to a swimming tadpole in only days) have made them favored model organisms in developmental biology. In particular, the African clawed frog, *Xenopus laevis*, has proven an invaluable tool from which to decipher the signaling pathways and networks that guide embryonic development. *Xenopus* embryos can endure extensive perturbations (for example tissue transplantations) and can be easily microinjected with diverse reagents either in the entire embryo or in a targeted manner (using
differences in pigmentation of dorsal or ventral fates) in order to activate or silence genes and pathways. The abundance of eggs provided by each female frog makes them an ideal source of cell-free extracts for *in vitro* studies, and makes high-throughput research practical.

Decades after Spemann’s observations were rewarded with the Nobel Prize for embryonic induction, the dawn of molecular biology and gene cloning allowed researchers to begin shedding light on the factors and biochemical pathways responsible for the organizer’s function and the embryo’s resilience. The first glimpse of Spemann organizer’s molecular nature was shown by *goosecoid*, a homeobox gene isolated from dorsal cDNA libraries of dissected dorsal blastopore lips of *Xenopus laevis*, which was specifically expressed in the Spemann organizer (Cho et al., 1991). Amazingly, *goosecoid* had the ability to induce a second axis when injected into ventral cells.

Since the Spemann organizer is able to affect the fate of neighboring cells, it seemed likely that mechanism accounting for its inductive properties relied on secreted factors. In the following years, functional screens allowed for the discovery of multiple genes encoding secreted factors that regulate patterning of the *Xenopus* gastrula. The expression of many of them was confined to the Spemann organizer. For example, Chordin, Noggin, and Follistatin were organizer-specific and were capable of inducing dorsal neural tissue when overexpressed (Smith and Harland, 1992; Hemmati-Brivanlou et al., 1994; Sasai et al., 1994).

In the following pages, the most important components of the extracellular network of interacting proteins that ensure the dorsal-ventral (DV) patterning in *Xenopus* are described (Fig 1-1). These secreted proteins form a circuit that creates and maintains the BMP morphogenetic signaling gradient that determines ventral versus dorsal fates (Fig. 1-2). The function of one of those proteins, Crescent, was revealed in the first part of this thesis.
Fig. 1-1. The Gastrula signaling centers. The dorsal Spemann organizer (blue) and the ventral center (red) express a cocktail of growth factors and growth factor antagonists that will form an network of interacting proteins that will determine dorsal-ventral patterning (Modified from De Robertis, 2006).
Fig. 1-2. A gradient of BMP signaling patterns DV polarity. This BMP gradient is read by cells and converted into C-terminal phosphorylations of SMAD1/5/8, the transcription factor in charge of transducing the BMP signal. Whole-mount immunostaining for phospho-SMAD1 allows the visualization of the BMP gradient, high in ventral and lower towards dorsal regions (From Plouhinec and De Robertis, 2009).
Secreted factors that ensure a resilient BMP signaling gradient:

**Chordin**

In *Xenopus*, Chordin (Chd) is first expressed in the dorsal ectoderm of the blastula and later in the Spemann organizer at gastrula. In later stages, it is expressed in the notochord and prechordal plate (Kuroda et al., 2004). Chordin has been shown to bind BMPs in the extracellular space and prevent BMP signaling (Piccolo et al., 1996). The BMP-binding modules of Chordin are comprised of four cysteine-rich domains (CRDs), also referred to as von Willebrand Factor-C (vWF-c) domains (Larrain et al., 2000). Other extracellular regulators of TGF-β signaling also contain these domains, such as Crossveinless-2 (CV-2) Procollagens, Amionless, Neuralin, Keilin, and Connective Tissue Growth Factor (CTGF) (Abreu et al., 2002). Contrary to other BMP antagonists, Chordin can be cleaved by tolloid metalloproteinases, which results in the release of intact BMP ligands and restoration of BMP signaling (Piccolo et al., 1997). In this way, Chordin can act as a shuttle for BMP, allowing BMP signaling at a distance from the Chordin source (Plouhinec et al., 2011). In the gastrula, Chordin diffuses along Brachet’s cleft, the extracellular space that separates ectoderm from endomesoderm which may bring both the ectoderm and mesendoderm under the control of the same BMP signaling gradient (Plouhinec et al., 2013). Chordin is arguably the most important factor secreted from the Spemann organizer, and is at the heart of the network of secreted proteins that produces the robust BMP signaling gradient that determines DV fates (Fig 1-3).
Fig. 1-3. A network of secreted proteins regulates the BMP gradient. DV patterning in *Xenopus* is regulated by a biochemical pathway of proteins secreted by the Spemann organizer and ventral center. These proteins create and maintain a robust BMP signaling gradient. Direct protein–protein interactions are indicated by black arrows, transcriptional regulations by blue arrows, and red arrows represent the flux of Chordin/BMP complexes. Chordin (Chd) binds BMP2/4/7 and ADMP, preventing BMP signaling dorsally, and transports these growth factors towards ventral regions, where Tolloid proteases (Xlr/BMP1), in cooperation with the scaffold protein Ont1, cleave Chd and release BMPs. The ventral center also secretes Sizzled, and CV2 important feedback regulators of BMP signaling. BAMBI and Twisted gastrulation (Tsg) have been omitted for simplicity (From Plouhinec and De Robertis, 2009).
Noggin

Noggin is another BMP antagonist secreted by the Spemann organizer at gastrula, and by the notochord and prechordal plate later in development (Smith and Harland, 1992). Similarly to Chordin, Noggin also inhibits BMP signaling by binding to BMPs and preventing ligand-receptor interaction (Zimmerman et al., 1996). The crystal structure of Noggin bound to BMP7 has been resolved. BMP7 homodimers are bound by a butterfly shaped dimer of Noggin, which blocks the molecular interfaces in BMP7 that normally bind to BMP receptors (Groppe et al., 2002). Structural similarities between Noggin and BMP ligands suggest that they may have evolved from a common ancestral gene (Groppe et al., 2002).

Follistatin

The Spemann organizer also secretes the BMP inhibitor Follistatin (Hemmati-Brivanlou et al., 1994). Originally discovered from follicular fluid of the porcine ovary, Follistatin owes its name to the fact that it had been shown to inhibit the secretion of follicle-stimulating hormone (FSH) from the anterior pituitary gland (a previous name was FSH-suppressing protein “FSP”) (Ueno et al., 1987). Follistatin is expressed in nearly all tissues of higher animals, and is involved in numerous physiological processes like inflammatory responses, skin regeneration, and folliculogenesis in the ovary. Follistatin binds TGF-βs (especially Activin) and BMPs growth factors, inhibiting signaling (Thompson et al., 2005; Harrington et al., 2006; Fainsod et al., 1997; Iemura et al., 1998). The anti-BMP activity of Follistatin is thought to regulate DV patterning (De Robertis and Kuroda, 2004).
**Xnr3**

*Xenopus* Nodal-related protein 3 (Xnr3) is a divergent member of the TGFβ superfamily expressed in the Spemann organizer. Although Nodal proteins are known to give rise to mesoderm, Xnr3 is an exception as it lacks mesoderm-inducing ability (Hansen et al., 1997). In the organizer, it may antagonize BMP signaling and induce neural differentiation (Haramoto et al., 2007). Later in development, Xnr3 affects brain patterning at the neural-epidermal boundary (Morita et al., 2013).

**sFRPs**

Secreted Frizzled Related proteins (sFRPs) are, as their name suggests, secreted forms of the Wnt receptor Frizzled (Fz) (Semenov and He, 2003). They lack however, the seven transmembrane and intracellular domains of Fz (Povelones and Nusse, 2005). sFRPs are the largest and most extensively studied family of Wnt modulators (Semenov and He, 2003). sFRP proteins can be structurally divided into an amino-terminal half, that contains the Fz domain (also called cysteine-rich (CRD) domain), and a carboxyl-terminal half which includes the Netrin (NTR) domain, whose function remains a mystery (Semenov and He., 2003). sFRPs modulate Wnt signaling by binding Wnts in the extracellular space through their CRD domain, also present in the Frizzled Wnt-receptor (Leyns et al., 1997; Kawano and Kypta, 2003). It is thought that many sFRPs inhibit Wnt signaling by impeding the interaction between Wnt and Frizzled. The Spemann organizer is a rich source of sFRPs, with Crescent, Frzb-1/sFRP3 and sFRP2 being the main ones expressed (Leyns et al., 1997; Wang et al., 1997). Microinjections of these sFRPs dorsalize *Xenopus* embryos, implying that the late Wnt pathway plays an important
role in the establishment of DV polarity (Pera and De Robertis, 2000; Shibata et al., 2000; Bradley et al., 2000). sFRPs can be divided, based on amino acid sequence similarity, into 3 subfamilies: sFRP1, Frzb-1, and a third family consisting of Crescent and Sizzled (Kawano and Kypta, 2003). The sFRP1 subfamily consists of sFRP1, sFRP2 and sFRP5, while the FrzB subfamily includes Frzb-1/sFRP3 and sFRP4. Crescent and Sizzled represent a distinct subfamily (Kawano and Kypta, 2003).

**Crescent** is an sFRP expressed in *Xenopus*, chicken, zebrafish, platypus and opossum. Crescent and Sizzled, an closely evolutionarily related sFRP, comprise a subfamily within the sFRPs that differs from the other subfamilies by the presence of an extra cysteine in the Fz CRD domain and absence of a cysteine in the NTR domain. Crescent was originally discovered as a cDNA expressed in the anterior endomesodermal crescent of the chick embryo (Pfeffer et al., 1997). Expression studies in *Xenopus* detected *Crescent* mRNA expressed in Spemann organizer at early gastrula, and later at stages in the prechordal plate and in the deep anterior endoderm (Pera and De Robertis, 2000; Shibata et al., 2000). Microinjections of *crescent* mRNA in *Xenopus* embryos causes dorsalized phenotypes (large heads and short trunks) (Ploper et al., 2011). Crescent also been shown to bind and inhibit Wnt-induced double axis formation.

**Frzb** stands for Frizzled motif associated with bone development. Although Frzb-1/sFRP3 is expressed in many adult mammalian tissues, in the *Xenopus* gastrula it is expressed dorsally by the Spemann organizer (Leyns et al., 1997). At midgastrula, Frzb-1/sFRP3 is expressed in the anterior involuting endomesoderm of the organizer, prospective foregut and prechordal plate.
Similarly to Crescent, Frzb-1/sFRP3 mRNA microinjections dorsalize *Xenopus* embryos and are capable of inhibiting Wnt8 activity in double axis formation assays (Leyns et al., 1997).

sFRP2 was isolated together with Crescent in a screen for of secreted antagonists isolated from *Xenopus* Spemann organizer (Pera and De Robertis, 2000). Although sFRP2 expression coincides with that of Crescent and Frzb-1/sFRP3 in the Spemann organizer at the onset of gastrulation, during neurula stage sFRP2 transcripts are copiously expressed in the neural plate but not in the midline (which gives rise to the floor plate).

**Dickkopf**

Dickkopf-1 (Dkk-1) is one of the main Wnt antagonists secreted by the Spemann organizer. The Dkk family consists of four evolutionary conserved secreted proteins (Dkk1-4) that inhibit Wnt signaling by antagonizing the Wnt coreceptor LDL receptor-related protein 5/6 (LRP5/6) (Glinka et al., 1998; Niehrs, 2006). Other studies have found that Dkk induces LRP5/6 endocytosis from the cell membrane in combination with a Dkk co-receptor called Kremen (Mao et al., 2002). In this manner, the interaction of Dkk with LRP5/6 prevents the binding of canonical Wnts to their receptors and regulates patterning not only of antero-posterior (AP) axis and head formation, but also of limb formation, somitogenesis, and eye development (Niehrs, 2006).
**Cerberus**

Cerberus is a secreted antagonist of TGFβ/Nodal, BMP, and Wnt pathways (Piccolo et al., 1999). In terms of structure, Cerberus is a cystine-knot protein of the eight-member ring DAN family, together with Gremlin, Coco, and DAN (Avsian-Krechmer and Hseuh, 2004). In the *Xenopus* gastrula, this multivalent inhibitor is expressed in the anterior dorsal endoderm, and is capable of inducing ectopic head structures (Bouwmeester et al., 1996).

As detailed above, the Spemann organizer is specialized in secreting a cocktail of growth factor antagonists, in particular inhibitors of the BMP, TGFβ and Wnt pathway. Other regulators of these pathways include Gremlin, another BMP inhibitor (Hsu et al., 1998), and Coco/Cerl2, a multivalent inhibitor of Nodal, Activin and BMP (Schwickert et al., 2010).

Although many secreted proteins and their function in DV patterning have been discovered in the past 20 years, there is still a possibility that many more remain undescribed. In support of this, high-throughput RNA sequencing data from different *Xenopus* experimental conditions (like dorsal or ventral halves) not only confirm the localization and regulation of the proteins previously described in this system, but also detect transcripts for unexpected and potentially novel regulators of the DV patterning (De Robertis lab, unpublished results). Studying and validating these new factors individually in the embryo will be required to confirm if they play a role in DV polarity. Undoubtedly, RNA sequencing from *Xenopus* will uncover novel players in the pathway that determines dorsal versus ventral specification.
ADMP

The anti-dorsalizing morphogenetic protein (ADMP) is a dorsally secreted BMP ligand (Moos et al., 1995). ADMP transcription is activated by low BMP signaling levels, explaining its dorsal expression, and providing a negative feedback loop for the activity of the Spemann organizer, which ultimately confers robustness to DV patterning in the developing gastrula (Reversade and De Robertis, 2005). Although knockdown of ADMP in the whole embryo causes only mildly dorsalized phenotypes that retaining DV polarity, knockdown in dorsal half embryos causes extreme dorsalizations (Willot et al., 2002; Reversade and De Robertis, 2005). ADMP is essential in creating a self-regulating *Xenopus* blastulae, and proves that the morphogenetic field requires not only ventral, but also dorsal, BMP sources.

Ont1

Olfactomedin-family factor 1 (Ont1), a member of the Olfactomedin family of secreted proteins, is a dorsally secreted scaffold for tolloid-mediated degradation of Chordin (Inomata et al., 2008). The Olfactomedin family of related proteins, which contain an amino terminal coiled-coiled domain and a conserved Olfactomedin domain towards it carboxyl terminus, comprise a group of secreted factors with elusive biological roles (Zeng et al., 2005; Tsuda et al., 2002). Ont1 was initially isolated from chick embryos, where it is expressed in the in the axial and paraxial mesoderm (Sakuragi et al., 2006). In *Xenopus* however, *ONT1* expression is initially ubiquitous but ultimately becomes localized to the organizer region during gastrulation, where it plays a key role in the regulation of DV patterning. By acting as a pro-BMP factor that assists in
the degradation of Chordin, Ont1 functions as a negative feedback regulator of the Spemann organizer (Inomata et al., 2008) (Fig. 1-3).

The Ventral Center

Although Hans Spemann has referred to the ventral region of the gastrula as being an “indifferent region” of the embryo (Spemann and Mangold, 1924), transplantation of ventral centers into dorsally-radialized embryos restores dorsal-ventral patterning, demonstrating the importance of the signals secreted by these ventral cells (Reversade and De Robertis, 2005). In the following pages, the factors secreted by the ventral center of the gastrulating Xenopus embryo are described.

BMP morphogens

BMP growth factors, in combination with inhibitory signals from the Spemann organizer, are the driving force behind DV patterning. Although BMP2, BMP4 and BMP7 transcripts are maternally deposited in the Xenopus oocyte, BMP signaling only begins to have effect during midblastula transition (Faure et al., 2000; Schohl and Fagotto, 2002). At midblastula transition, BMP4 and BMP7 are expressed throughout the entire embryo. However, their expression begins to be excluded in dorsal regions (Fainsod et al., 1994; Dale et al., 1992; Wang et al., 1997). The transcription of BMP4 and BMP7 genes are activated by BMP signaling, constituting a positive feedback loop, which reinforces high ventral BMP transcription, secretion and signaling (Metz et al., 1998) (Fig. 1-3). Phosphorylated SMADs, transcription factors in charge of transducing BMP
signals, provide a direct readout of BMP activity and allow for visualization of the BMP gradient (Fig. 1-2). The expression and regulation of these BMPs constitute one of the foundations of the BMP signaling gradient that patterns the DV axis.

**BAMBI**

The BMP and Activin membrane-bound inhibitor (BAMBI) is expressed ventrally in the in the *Xenopus* gastrula. As its name indicates, BAMBI is a transmembrane protein, and it resembles a type I BMP receptor that lacks the intracellular catalytic domain. This allows BAMBI to function as a dominant-negative BMP receptor, by binding BMP and Activin proteins without signalling towards the BMP signaling pathway, thus hindering BMP signaling on the ventral pole (Onichtchouk et al., 1999). Since BAMBI is transcriptionally activated by BMP signaling, it functions as a negative feedback regulator of BMP signaling on the ventral side of the gastrula (Reversade and De Robertis, 2005).

**Xlr**

Xolloid-related (Xlr) is a ventrally expressed tolloid metalloprotease secreted in the developing *Xenopus* gastrula (Fig 1-3). Two other tolldoids secreted in the gastrula, although ubiquitously, are BMP1 and Xolloid (Goodman et al., 1998; Dale et al., 2002). Xolloid stands for “*Xenopus* tolloid”, in reference to a *Drosophila* gene that regulates DV patterning (Piccolo et al., 1997). BMP1 was the first tolloid metalloprotease to be isolated from vertebrates, and owes its name to the fact that it originally copurified with other BMP growth factors purified from bone extract (Wozney and Rosen, 1988). Tolloid enzymes are zinc metalloproteinases of the astacin
family (Bond and Beynon, 1995) that cleave many extracellular substrates, such as procollagens, small leucine-rich proteoglycans, and lysyl oxidase (Ge and Greenspan, 2006). In the *Xenopus* gastrula, tolloids regulate DV patterning by proteolytically cleaving Chordin at two specific sites (Piccolo et al., 1997; Marques et al., 1997; Scott et al., 1999).

By cleaving Chordin, tolloids inactivate the main BMP inhibitor of the DV patterning network, causing the liberation of intact BMP growth factors. In this way, tolloids act as pro-BMP factors. Inactivating mutations in tolloids can cause dorsalized phenotypes, as in the case of the Zebrafish *minifin* mutant, in which the Tolloid-like1 gene is mutated (Connors et al., 1999). The *minifin* phenotype is thought to be predominantly caused by defects in Chordin regulation, since fish double mutant for both Tolloid-like1 (*minifin*) and Chordin (*chordino*) exhibit similar phenotypes to those of *chordino* mutants (Wagner and Mullins, 2002).

**CV2**

Crossveinless-2 (CV2) is a ventrally-secreted factor, structurally related to Chordin that contains five cysteine-rich domains (CRDs), and a partial von Willebrand Factor-D (vWF-d) domain. The *Drosophila* homologue is required for the formation of crossveins in the developing wing. Although CV2 binds BMPs with high affinity (Rentzsch et al., 2006, Zhang et al., 2007), the net effect on BMP signaling can vary, with some studies showing CV2 as a pro-BMP (Conley et al., 2000; Ralston and Blair., 2005; Ikeya et al., 2006; Rentzsch et al., 2006; O’Connor et al., 2006), while others finding CV2 to have anti-BMP effects (Moser et al., 2003; Coles et al., 2004; Binnerts et al., 2004; Zhang et al., 2007). In the *Xenopus* gastrula, CV2 is transcribed ventrally by high BMP levels, where it acts as al local feedback inhibitor of BMP
signaling by binding BMPs (Ambrosio et al., 2008). CV2 is thought to remain tethered to cells in which it was produced, through binding of its vWF-d domain to glypicans attached to the plasma membrane (Serpe et al., 2008). Additionally, CV2 triggers the clearance of BMPs from the extracellular space through an endocytic trap mechanism (Kelly et al., 2009).

CV2 binds Chordin with high affinity, and binds Chordin-BMP complexes with even higher affinity. A model for the pro-BMP activity of CV2 has been proposed in which CV2 functions as a molecular sink for Chordin-BMP complexes on the venrtal side, concentrating Chordin-BMP complexes made in more dorsal regions (Fig. 1-3). In this region, tolloid proteolytic cleavage of Chordin would liberate BMPs from these complexes and allow peak BMP signaling at ventral-most regions of the gastrula (Zakin and De Robertis, 2010).

**Tsg**

The ventral center expresses twisted gastrulation (Tsg), a protein whose homologue in *Drosophila* was first shown to affect gastrulation (Zusman and Wieschaus, 1985). In *Xenopus*, Tsg can have both pro- or anti-BMP activities. Although Tsg can bind to both Chordin and BMP creating a ternary complex that impedes the binding BMP ligand to its receptor and inhibiting BMP signaling, it also assists in tolloid-mediated cleavage of Chordin, promoting BMP signaling (Oelgeschlager et al., 2000; Chang et al., 2001; Larrain et al., 2001). Tsg is thought to keep BMPs in a soluble state, as BMP ligands have a strong tendency to bind extracellular matrix proteins. The effect of Tsg on BMP signaling, and thus on the gradient that determines DV patterning, seems to be greatly dependent on dose. In *Xenopus* embryos, low levels of Tsg mRNA injections cause dorsalized phenotypes, while larger amounts result in ventralizations
Oelgeschlager et al., 2003; Ross et al., 2001). Tsg knock-down experiments in zebrafish show that CV2 has an overall pro-BMP function (Blitz et al., 2003; Little and Mullins, 2004; Xie and Fisher, 2005; Zakin et al., 2005).

**Sizzled**

Sizzled is a ventrally expressed sFRP that plays a vital role in DV patterning. Sizzled was originally identified by genetic screens in zebrafish as a ventralizing mutation and named ogon, (previously also known as mercedes) (Hammerschmidt and Mullins, 2002). The ogon mutation localized to the locus of an sFRP gene named Sizzled, for secreted frizzled (Yabe et al., 2003; Martyn and Schulte-Merker, 2003). Unlike other sFRPs, Sizzled does not inhibit Wnt signaling (Collavin and Kirschner, 2003 and Yabe et al., 2003). Overexpression of Sizzled results in dorsalized embryos, and this dorsalizing ability requires Chordin (Salic et al., 1997; Yabe et al., 2003).

Although sFRPs are generally recognized as modulators of the Wnt pathway, Sizzled acts as an anti-BMP factor in the developing gastrula. Sizzled competitively inhibits tolloid proteinases that cleave and inactivate Chordin (Lee et al., 2006) (Fig. 1-3). In this manner, Sizzled stabilizes Chordin and causes inhibition of BMP signaling. Sizzled expression is activated by BMP signaling on the ventral pole of the gastrula, and serves as a negative feedback inhibitor of BMP signaling (Lee et al., 2006; Muraoka et al., 2006). Additionally, Sizzled has been recently shown to be an expander molecule required for robust patterning and scaling of the BMP gradient in *Xenopus* embryos (Inomata et al., 2013; Bier and De Robertis, 2015). Evolution
has repurposed the ventrally expressed sFRP Sizzled from a Wnt inhibitor to a tolloid inhibitor and from the Wnt to the BMP pathway (Mullins, 2006).

sFRPs in the BMP pathway

After Sizzled was uncovered as a tolloid inhibitor that functioned in the BMP pathway, one question that immediately followed was whether Crescent, its dorsally expressed counterpart, regulated the Chordin-BMP pathway in a similar manner. If Sizzled functioned as a negative feedback regulator of BMP signaling in the ventral center, what then is the role of Crescent that is expressed dorsally? Specifically, what is the relationship between these two evolutionarily related sFRPs expressed on opposite poles of the embryo? Crescent had been known to bind and modulate Wnt signaling. Was the phenotype of Crescent entirely due to its anti-Wnt effects? Chapter 2 of this thesis addresses these questions.
Introduction Part II

Crosstalk between the Wnt and endocytosis pathway

In this section I introduce Wnt/GSK3 regulation of protein stability and its effects on the MiT family of transcription factors
Wnt signaling and requires endocytic/multivesicular body (MVB) pathway

Canonical Wnt signaling influences tissue differentiation, growth, and homeostasis by stabilizing the transcriptional activator β-Catenin (McDonald et al., 2009; Angers and Moon, 2009). Dysregulation of Wnt pathway components lead to improper development and cancer, among other diseases (Clevers and Nusse, 2012). The mechanism of Wnt signaling consists in inhibition of a destruction complex composed of adenomatous polyposis coli (APC), Axin1, casein kinase 1α (CK1α), and glycogen synthase kinase 3 (GSK3) (Cadigan and Peifer, 2009). In the absence of Wnt ligands, GKS3 bound to the destruction complex phosphorylates β-Catenin, triggering its polyubiquitination and degradation. However, upon Wnt ligand stimulation the destruction complex is inhibited and newly synthesized β-Catenin is allowed to accumulate, translocate in the nucleus, and regulate transcription of Wnt-responsive genes (Peifer et al., 1994).

The Wnt signal transduction requires the endosomal sorting complexes required for transport (ESCRT) machinery in order to inhibit the destruction complex (Taelman et al., 2010; Dobrowolski et al., 2011; Vinyoles et al., 2014). Wnt stimulation of its receptor low-density lipoprotein receptor-related protein 6 (LRP6) recruits the destruction complex, including Axin1, GSK3, and phospho-β-Catenin (p-β-Catenin), and is subsequently endocytosed as membrane-bound signaling organelles called “Wnt/LRP6 signalosomes” (Bilić et al., 2007). Wnt signalosomes are then trafficked from the cytosol into intraluminal vesicles of multivesicular bodies (MVBs) (Taelman et al., 2010; Vinyoles et al., 2014). In this way, the destruction complex containing GSK3 and Axin1 is separated from its cytosolic substrates by double membranes organelles.
Recent studies have shown that Wnt signaling stabilizes many other proteins in addition to β-Catenin (Taelman et al., 2010; Acebron et al., 2015). The basis of this effect on protein stability is that GSK3 phosphorylates a myriad of cellular proteins, generating phosphodegrons which are then recognized by E3 ubiquitin ligases that trigger polyubiquitination of these proteins (Acebron et al., 2014). Polyubiquitinated proteins are subsequently targeted for proteosomal degradation (Acebron et al., 2014). This mechanism, recently named “Wnt-dependent STabilization Of Proteins” or Wnt/STOP, is considered independent of β-Catenin-driven transcriptional effects and is emerging as an important mechanism within canonical Wnt signaling (Acebron et al., 2014).

The GSK3 proteome

Glycogen synthase kinase 3 (GSK3) plays pivotal roles in regulating cellular catabolism. GSK3 phosphorylates a myriad of proteins in a processive manner, and prefers pre-phosphorylated substrates (Cohen and Frame, 2001). Among its most famous targets are Glycogen Synthase, β-Catenin, HIF1α, Tau, CREB and c-Myc (Sutherland, 2011). GSK3 has been shown to target substrates such as β-Catenin for polyubiquitination and subsequent proteasomal degradation (McDonald et al., 2009). In this effect lays the basis for canonical Wnt signaling. In the absence of Wnt stimulation, β-Catenin levels are kept at bay by constitutive GSK3 activity, which marks β-Catenin for proteolysis in the proteasome. However, upon Wnt ligand stimulation, GSK3 is sequestered into multivesicular endosomes, also known as multivesicular bodies (MVBs), resulting in the stabilization of newly synthesized β-Catenin,
which promptly accumulates in the nucleus and exerts its transcriptional effects by modulating the transcription of Wnt-responsive genes (Taelman et al., 2010).

Wnt signaling has been shown to stabilize many proteins in addition to β-Catenin by decreasing the polyubiquitination triggered by the generation of GSK3-induced phosphodegrons (Kim et al., 2009; Taelman et al., 2010; Acebron et al., 2014). This Wnt/STOP branch of Wnt signaling surely has broad and yet unknown effects on cellular physiology (Acebron et al., 2014). Some proteins stabilized by Wnt signaling function as important nodes for signaling by integrating the Wnt signal into other pathways, e.g. Smad1 and Smad4, effectors of the BMP pathway that are GSK3 substrates and stabilized at the protein level by Wnt signaling (Fuentealba et al., 2007; Demagny et al., 2014). The resulting crosstalk between the BMP and Wnt pathway is important in development as it enables any given cell in the embryo to read two parameters: the intensity and the duration of the activated SMAD signal (Fuentealba et al., 2007; Eivers et al., 2008).

In an attempt to predict and discover new proteins whose stability might be regulated by Wnt signaling, a bioinformatic screen of the human proteome was carried out. By screening for putative GSK3 targets containing three or more consecutive phosphorylation sites followed by a priming site revealed, it was determined that an astonishing 20% of human proteins contain these putative phosphorylation sites (Taelman et al., 2010). Among all proteins, the highest score in terms of phylogenetical conservation of the GSK3 sites belongs to the MiT family of basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factors, providing the starting point for the fourth chapter of this thesis (Figure 1-4).
Fig. 1-4. The MITF family members are putative GSK3 substrates. A short list of putative GSK3 targets as determined by a bioinformatic screen of the human proteome (Taelman et al., 2010). The MiT family (MITF/TFE3/TFC/TFE3), indicated in red, scored highest in the phylogenetic conservation index of putative GSK3 sites.
**The MiT Family**

The Microphthalmia-associated transcription factor (MiT) family defines an evolutionarily conserved group of basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factors with important roles in development, organelle biogenesis and cellular homeostasis. Dysregulation of these transcription factors leads to disease (Haq and Fisher, 2011; Fergusson 2015; Ploper and De Robertis, 2015). The family consists of four members, MITF, TFEB, TFE3 and TFEC. Structurally, these proteins contain a HLH domain through which they can homo and heterodimerize with each other (but not with other bHLH proteins), a basic domain which is used for binding target DNA sequences containing Ephrussi box (E-Box) motifs [CAC(G/A)TG], and an activation domain by which they regulate gene transcription. They also contain a highly conserved serine-rich region towards the C-terminus, which has only recently being recognized as important for posttranslational regulations (Steingrimsson et al., 2004; Ferron et al., 2013; Ploper et al., 2015).

The roles of MiT transcription factors in adaptation to starvation have been highly conserved throughout evolution (Martina et al., 2014). HLH-30, the only MiT ortholog found in *C. elegans*, plays analogous roles to its mammalian counterparts. In response to starvation, HLH-30 accumulates in the nucleus, binds to DNA sequences that resemble elements found in promoters of lysosomal genes, and drives the transcription of lysosomal lipases and autophagy genes (Grove et al., 2009; Lapierre et al., 2013; O’Rourke and Ruvkun, 2013; Settembre et al., 2013). Developmental functions of MiT proteins have also been conserved in evolution, as the *Drosophila* MITF homolog, like its mouse counterpart, is required for eye development and is
transcriptionally regulated by Pax6 (Eyeless) (Hallsson et al., 2004). In mammals, MiT members have been shown to participate in a broad range of cellular processes, from stem cell differentiation (Betshinger et al., 2013) to nutrient sensing and energy homeostasis (Martina et al., 2014), to genomic stability and DNA repair (Strub et al., 2011).

Given that MiT transcription factors can bind the same E-box [CAC(G/A)TG] DNA sequence, functional redundancy has been observed between them (Steingrimsson et al., 2002). They share many downstream targets, and it seems likely that significant crosstalk and compensation can occur among members of this family. However, while MITF is required for development and survival of melanocytes, TFE3 null mice have no phenotype and TFEB null mice are embryonic lethal due to defects in placental vascularization (Steingrimsson et al., 1998; Steingrimsson et al., 2002), suggesting that MiT redundancy and compensation mechanisms might not be functional in every tissue or developmental stage.

In humans, overexpression of MiT members due to genomic amplifications, chromosomal translocations or promoter swapping, are frequently associated with diseases such as melanoma, clear cell and alveolar soft part sarcoma, and renal cell carcinoma (Haq and Fisher., 2011). MITF, TFE3, and TFEB overexpression have all been observed in renal cell carcinomas (Haq and Fisher, 2011; Kauffman et al., 2014), where they are required for transformation and can compensate for each other, arguing that at least in this disease model, they may be activating the same genes and promoting oncogenesis through similar mechanisms.

MITF

In 1942, Paula Hertwig described the original white-coated microphthalmia mutant mice (Hertwig, 1942). Fifty years later, the mitf gene was cloned from mice using a gene-trap insertion
in the *mitf* promoter of *mitf^{ex9/vga9}* (Hodgkinson et al., 1993). The *microphthalmia* locus was associated with mutations in a gene encoding a novel bHLH-LZ protein. Mutations of *mitf* perturb the development of several cell lineages, including osteoclasts, mast cells, retinal pigment epithelium (RPE) and most notably, melanocytes (Steingrimsson et al., 2004; Arnheiter et al., 2007). A wide range of phenotypes are associated with *mitf* mutations. They can range from hypomorphic to dominant negative, leading to variations in coat color, hearing defects, and bone and mast cell disorders. MITF null mice have complete absence of melanocytes in their skin, eye, and ear, resulting in white, blind and deaf animals (Lerner et al., 1986; Steingrimsson et al., 2004; Arnheiter et al., 2007). MITF is a very conserved protein, both in structure and in function, and has been detected throughout all vertebrate species investigated, in addition to the ascidian *Halocynthia roretzi*, *Caenorhabditis elegans* and *Drosophila melanogaster* (Rehli et al., 1999; Tachibana, 2000; Yajima et al., 2003; Hallsson et al., 2004).

The *mitf* gene is expressed in a wide variety of tissues, mainly in melanocytes, osteoclasts, retinal pigment epithelium (RPE), as well as in mast cells, natural killer cells, macrophages, B cells, and cardiac muscle. MITF activates the expression of a different subset of genes in each of these different cell types (Steingrimsson et al., 2004). Several isoforms of MITF have been described, some of which are tissue-specific. These different isoforms arise from differential promoter usage and differential splicing (Arnheiter et al., 2007). The main difference between isoforms resides in the first exon (Steingrimsson et al., 2004). MITF-M, is an isoform preferentially transcribed in melanocytes, while MITF-D is mainly expressed in RPE and monocytes. MITF-Mc appears to be exclusively employed by mast cells, while MITF-A and MITF-H are more ubiquitously expressed, though at low levels, throughout many tissues (Takeda et al., 2002; Takemoto et al., 2002; Steingrimsson et al., 2004; Arnheiter et al., 2007).
Undoubtedly, melanocytes and melanoma, which express MITF-M, have been the preferred tissue for studying the roles of MITF. Melanocytes are pigment-producing cells localized in diverse organs of the body, including epidermis, hair follicles, inner ear and eyes, and are specialized in synthesizing melanin (Steingrimsson et al., 2004). This pigment gives color to skin, hair and eyes and protects against ionizing radiation, and is synthesized and transported in membrane-bound organelles known as known as melanosomes (Raposo and Marks, 2007).

MITF-M is as a master gene regulator of melanocytes, required for the development, proliferation and survival of this lineage (Arnheiter et al., 2007). MITF is detected in melanoblasts, where it serves as a marker of this migrating neural crest population. In this lineage, transcription of mitf-m is activated by Pax3, Sox10, CREB, and Lef-1 through the melanocyte-specific mitf-m promoter. These factors ensure a cell-specific, temporally-regulated, and cAMP-activated expression of MITF-M (Steingrimsson et al., 2004; Arnheiter et al., 2007).

The MITF-M is a constitutively nuclear transcription factor that regulates the expression of a myriad of different genes. Particularly, it activates the expression of a large subset of melanogenic enzymes required for synthesizing melanin such as tyrosinase (TYR), tyrosinase related protein 1 (TRP1), dopachrome tautomerase (DCT), as well as MART1 and MLANA, genes required for the formation of the melanosomal matrix (Cheli et al., 2010). However, numerous studies which include RNA-seq and ChIP-seq data have shown that MITF also regulates the transcription and/or binds to the promoter of many genes involved in cell cycle, apoptosis, and DNA damage response (Strub et al., 2011; Laurette et al., 2015). The suite of genes activated by any given MITF isoform seems to be highly dependent on the cellular context and presence of additional cofactors (Arnheiter et al., 2007). MITF had been reported to drive
transcription of target genes by binding a specific sequence named the “M-Box” (5’-AGTCATGTGCT-3’) (Lowings et al., 1992; Bertolotto et al., 1998). However, ChIP-seq data that revealed more general MITF binding motifs in a genome-wide manner, found MITF preferentially bound to sequences containing the E-boxes 5’-CACGTG-3’ and 5’-CATGTG-3’, and confirmed the requirement for a flanking T adjacent to the 5’ end of the E-box (Aksan and Goding 1998; Strub et al., 2011).

The MITF protein is highly regulated at the posttranslational level. Many of the pathways that control these modifications in MITF protein are important in melanocyte development and function (Steingrimsson et al., 2004; Arnheiter et al., 2007). The c-KIT ligand (also known as “stem cell factor” or “Steel factor”) activates MAPK signaling through activation of the c-KIT receptor, and leads to dual phosphorylations of MITF on serine 73 (S73) by ERK1/2 and serine 409 (S409) by p90/RSK1, which is itself activated by ERK1/2 (Fig. 1-5) (Wu et al., 2000; Arnheiter et al., 2007). The S73 ERK1/2-induced phosphorylation plays a role in increasing MITF transcriptional activity by promoting the binding of MITF to the CBP/p300 transcriptional coactivator (Sato et al., 1997; Price et al., 1998), but does not affect DNA binding (Wu et al., 2000). Importantly, phosphorylations at S73 and S409 render MITF protein less stable. This is thought to be mediated by proteasomal degradation due to increased polyubiquitination at lysine 201 (K201) (Xu et al., 2000) (Fig. 1-5). The role of S73 phosphorylation in vivo remains enigmatic, since mice with knock-in mutations of MITF<sup>S73A</sup> lack any melanocytic phenotype (Bauer et al., 2009). S409 has also been shown to be important for binding the SUMO E3 ligase PIAS3, which results in decreased transcriptional activity (Levy et al., 2003), although it is unclear whether phosphorylation of this residue affects MITF activity by blocking PIAS3 binding and PIAS3-mediated SUMOylation.
Fig. 1-5. Posttranslational modifications of MITF protein. The MAPK/ERK pathway phosphorylates S73 and S409 in melanocytes/melanoma and S307 in osteoclasts. In myeloid precursors MITF is phosphorylated at S173 by C-TAK1, promoting cytoplasmic localization. MITF is SUMOylated at K182 and K316 by the SUMO E2 and E3 ligases UBC9 and PIAS3, respectively, which regulate transcriptional activity. UBC9 had originally been reported as the ubiquitin ligase for ubiquitination at K201. USP13 deubiquitinates MITF and inhibits its proteasomal degradation. In chapter 4 of this thesis, novel C-terminal GSK3 phosphorylations (in red).
MITF is SUMOylated at lysine 182 (K182) and lysine 316 (K316), and this requires SAEI/SAEII and UBC9, SUMO E1 and E2 ligases (Murakami and Arnheiter, 2005) (Fig. 1-5). SUMOylation of MITF only regulates transcriptional activity, while dimerization, localization, DNA binding remain unaffected (Miller et al., 2005). Ubiquitination is also clearly important for MITF function. Although the ubiquitin E3 ligase involved for MITF remains elusive, the deubiquitinase USP13 can deubiquitinate MITF, regulate MITF stability and is essential for melanoma growth (Zhao et al., 2011).

MITF is essential for osteoclast development (Hershey and Fisher, 2004). In myeloid precursors, which give rise to osteoclasts, MITF is phosphorylated at S173 by Cdc25C-associated kinase (C-TAK)1 (Fig. 1-5). This modification increases interaction of MITF with 14-3-3 proteins and cytoplasmic retention, thus inhibiting MITF activity (Bronisz et al., 2006). During osteoclast differentiation, MITF is phosphorylated at serine 307 (S307) by the MAPK p38 in response to receptor activator of nuclear factor kappa-B ligand (RANKL) stimulation (Fig. 1-5). This modification increases MITF activity by promoting the formation of a trimeric complex with FUS and BRG1 (Mansky et al., 2002; Bronisz et al., 2013). Although this modification has yet to be described in melanocytes, MITF and BRG1 have been recently shown to interact and co-occupy regulatory elements in melanoma cells (Laurette et al., 2015). Despite these extensive investigations on MITF posttranslational modifications, close inspection of conserved sequences and domains allow for identification of previously unrecognized phosphorylation sites, such as the ones described in Chapter 4 of this thesis.
**TFEB and TFE3**

The transcription factors EB (TFEB) and E3 (TFE3) are key proteins that regulate lysosomal biogenesis, autophagy, and other cellular clearance pathways (Settembre et al., 2013; Martina et al., 2014). In addition, TFEB transcriptionally regulates lysosomal exocytosis, inducing both docking and fusion of lysosomes with the plasma membrane (Medina et al., 2011) TFEB and TFE3 are present in most, but not all, human tissues (Roman et al., 1991; Steingrimsson et al., 1998), and activate the expression of lysosomal and autophagosomal genes by binding Coordinated Lysosomal Expression And Regulation elements (CLEAR elements) in the gene promoters (Sardiello et al., 2009; Martina et al., 2014).

The activity of TFEB and TFE3 transcription factors is highly regulated by the mechanistic Target of Rapamycin Complex 1 (mTORC) pathway, which senses nutritional status at the outer lysosomal membrane and phosphorylates TFEB (and presumably TFE3) in nutrient rich conditions (Roczniak-Ferguson et al., 2012; Settembre et al., 2012; Martina et al., 2014). TFEB and TFE3 are recruited to the lysosome by interacting with Rag guanosine triphosphatases (GTPases) through their N-terminal domain (Martina and Puertollano, 2013). These phosphorylations retain TFEB and TFEB in the cytoplasm by promoting their binding to 14-3-3 cytosolic chaperones, thus limiting their nuclear accumulation and activity. When cells are subject to starvation, mTOR inhibition or lysosomal stress, mTOR dissociates from the lysosome, and unphosphorylated TFEB and TFE3 are free to accumulate in the nucleus and bind CLEAR elements and coordinately activate gene programs that will restore homeostasis, in addition to promoting the transcription of more tf eb through a positive feedback loop (Roczniak-Ferguson et al., 2012; Settembre et al., 2012; Settembre et al., 2013; Martina et al., 2014).
Recently, TFEB was shown to be dephosphorylated by calcineurin in response to lysosomal calcium signals, stimulating TFEB nuclear translocation (Medina et al., 2015).

Similarly to TFEB and TFE3, MITF-A and MIT-D isoforms are also subject to nutrient-driven and mTORC-regulated cytoplasmic retention (Roczniak-Ferguson et al., 2012; Martina and Puertollano, 2013). MITF-M however, the isoform expressed in melanocytes and considered a melanoma oncogene, escapes this mTORC-mediated inhibition as it lacks the amino-terminal Rag GTPase binding-domain required for lysosomal docking, which is present in all other MITF isoforms as well as TFE3 and TFEB.

**TFEC**

Despite the extensive research and literature on MITF, and the growing interest discoveries concerning TFEB and TFE3, the MiT member TFEC has not been extensively studied, perhaps due to its macrophage-restricted expression. Although the rat and mouse homologues were first reported to act as dominant negative regulators for other MiT factors due to the absence of an activation domain, the human TFEC does contain this domain and is capable of driving the expression, albeit weekly, of gene reporters for the tyrosinase or heme oxygenase-1 promoters (Rehli et al., 1991; Zhao et al., 1993; Rehli et al., 1999). The lack phenotype and the few changes in gene expression observed in TFEC-deficient macrophages, suggest that the role of TFEC in this lineage may be shared by other MiT factors (Rehli et al., 2005).

**Novel GSK3 phosphorylations in MiT transcription factors**

The MiT family ranked highest among all human proteins in terms of phylogenetic conservation of putative GSK3 phosphorylations (Fig. 1-4) (Taelman et al., 2010). Given that
this family of transcription factors play important roles in homeostasis, cellular differentiation and behave as oncogenes, finding additional signaling pathways and modifications which modulate the MiT family seemed important (Settembre et al., 2011; Steingrimsson et al., 2004; Medendorp et al., 2007; Haq and Fischer, 2011).

In chapter 4 of this dissertation, a study on MITF and Wnt signaling is presented. Of the 4 MiT factors, MITF was selected for further study because phospho-S409, the priming site for these putative GSK3 phosphorylations, was validated and known to render MITF less stable (Wu et al., 2000). A phosphospecific antibody directed against the two first GSK3 phosphorylation sites was generated (pMITF\(^{GSK3}\)), and proved that MITF was indeed phosphorylated in those residues by GSK3 (Ploper et al., 2015) (Fig. 1-5). Furthermore, these serines were revealed as important for MITF stability, and Wnt signaling was shown to stabilize MITF protein (Ploper et al., 2015). Additionally, MITF was shown to be a significant driver of endolysosomal biogenesis in melanomas. This was a novel and significant finding. Although TFEB and TFE3 had been established as key transcriptional regulators of lysosomal biogenesis pathways, MITF had never been implicated in lysosomal biogenesis. The late endolysosomes induced by MITF were not functional in proteolysis but, similarly to chloroquine-mediated lysosomal inhibition, enhanced Wnt signaling by increasing the sequestration of destruction complex components inside MVBs (Dobrowolski et al., 2012; Ploper et al., 2015). A model was presented in which Wnt induces stabilization of MITF protein, which in turn drives the generation of MVBs that enhance Wnt signals, and provides a positive feedback loop in the proliferative stages of melanoma growth (Ploper et al., 2015).
Chapter 2

Dorsal-Ventral patterning: Crescent is a dorsally secreted Frizzled-related protein that competitively inhibits Tolloid proteases

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Ploper, D., Lee, H.X., De Robertis, E. M.
Co-injections of Chordin and Crescent proteins into the blastocele of *Xenopus* embryos cause a massive expansion of the forebrain marker *Rx2a*, which is not observed when either component is injected alone. This cooperative effect between Chordin and Crescent on *Rx2a* expression is also not observed when co-injecting a point mutant Crescent that abolished its tolloid metalloproteinase inhibiting activity, but still retains its anti-Wnt effects. Thus, the synergistic interaction between Chordin and Crescent is due to the inhibition of Tolloid enzymes by Crescent protein (Ploper et al., 2011).
ABSTRACT

In *Xenopus*, dorsal-ventral (D-V) patterning can self-regulate after embryo bisection. This is mediated by an extracellular network of proteins secreted by the dorsal and ventral centers of the gastrula. Different proteins of similar activity can be secreted at these two poles, but under opposite transcriptional control. Here we show that Crescent, a dorsal protein, can compensate for the loss of Sizzled, a ventral protein. Crescent is a secreted Frizzled-Related Protein (sFRP) known to regulate Wnt8 and Wnt11 activity. We now find that Crescent also regulates the BMP pathway. Crescent expression was increased by the BMP antagonist Chordin and repressed by BMP4, while the opposite was true for Sizzled. Crescent knock-down increased the expression of BMP target genes, and synergized with Sizzled morpholinos. Thus, Crescent loss-of-function is compensated by increased expression of its ventral counterpart Sizzled. Crescent overexpression dorsalized whole embryos but not ventral half-embryos, indicating that Crescent requires a dorsal component to exert its anti-BMP activity. Crescent protein lost its dorsalizing activity in Chordin-depleted embryos. When co-injected, Crescent and Chordin proteins greatly synergized in the dorsalization of *Xenopus* embryos. The molecular mechanism of these phenotypes is explained by the ability of Crescent to inhibit Tolloid metalloproteinases, which normally degrade Chordin. Enzyme kinetic studies showed that Crescent was a competitive inhibitor of Tolloid activity, which bound to Tolloid/BMP1 with a $K_D$ of 11 nM. In sum, Crescent is a new component of the D-V pathway, which functions as the dorsal counterpart of Sizzled, through the regulation of chordinases of the Tolloid family.
INTRODUCTION

The early embryo has the capacity of self-regulating pattern. For example, when an amphibian embryo is bisected in such a way that both halves contain a part of the dorsal Spemann organizer, each half can regenerate a complete embryo and give rise to identical twins. Similarly, transplantation of dorsal organizer tissue into the ventral side of the gastrula embryo can generate a second morphogenetic field, resulting in the formation of Siamese twins (Spemann, 1938; De Robertis, 2006). In Xenopus and zebrafish, this remarkable inductive capacity is mediated by a network of interacting secreted proteins that establishes a gradient of Bone Morphogenetic Protein (BMP) signaling along the D-V axis (Little and Mullins, 2006; De Robertis, 2009). The Spemann organizer, where BMP signaling level is lowest, secretes the BMP antagonists Chordin, Noggin and Follistatin along with two BMPs, ADMP and BMP2. In the ventral center, genes such as Crossveinless-2 (CV2), BMP4, BMP7 and Sizzled are expressed in regions of high BMP signaling. This reciprocal transcriptional control at opposite poles helps explain self-regulation (De Robertis, 2009).

Chordin is a key D-V regulator secreted in large amounts by dorsal organizer tissue (Lee et al., 2006). Chordin binds to BMPs in the extracellular space and prevents them from binding to their cognate receptors, thus preventing signaling. Chordin/BMP complexes formed in more dorsal regions of the embryo are transported to ventral regions, where BMP ligands are released from inactive Chordin/BMP complexes by the cleavage of Chordin at two specific sites by Tolloid proteinases (Piccolo et al., 1997). This cleavage is facilitated by Ont-1, a scaffold protein of the Olfactomedin family that brings together Tolloid and its substrate Chordin (Inomata et al., 2008). Mathematical modeling suggests that the dorsal to ventral flux of Chordin/BMP provides
robustness to the system (Ben-Zvi et al., 2008; Plouhinec and De Robertis, 2009). The Chordin/BMP/Tolloid/CV2 network is an evolutionarily conserved biochemical pathway that regulates D-V patterning in many invertebrates, including *Drosophila*, and vertebrates (Little and Mullins, 2006; De Robertis, 2008; Umulis et al., 2009).

Extracellular regulation of growth factor signals is a common theme in embryonic patterning (Zakin and De Robertis, 2010). In addition to Chordin, many other growth factor inhibitors are produced in the *Xenopus* gastrula, such as the BMP inhibitors Noggin (Zimmerman et al., 1996), Follistatin (Hemmati-Brivanlou et al., 1994), and Gremlin (Hsu et al., 1998), and Wnt inhibitors such as Dickkopf (Dkk, an LRP6 inhibitor) (Glinka et al., 1998) and the sFRPs Frzb, sFRP2, Sizzled and Crescent (De Robertis and Kuroda, 2004). Multivalent inhibitors, such as Cerberus, which antagonizes Nodal, BMP and Wnt, and Coco/Cerl2, which inhibits Nodal, Activin and BMP, are also secreted (Belo et al., 2009; Schweickert et al., 2010). CV2 is expressed ventrally, where it avidly binds Chordin and Chordin/BMP complexes, serving as a sink for the continuous flow of dorsally secreted molecules towards the ventral center (Ambrosio et al., 2008; Kelly et al., 2009).

sFRPs contain Frizzled Wnt-binding domains and antagonize Wnt signaling by preventing their binding to Frizzled receptors (Leyns et al., 1997; Shibata et al., 2005). Structural predictions suggest that the Frizzled domains in sFRPs may recognize lipid modifications present in Wnts (Willert et al., 2003; Bazan and de Sauvage, 2009). Some sFRPs have also been shown to enhance Wnt signaling (Uren et al., 2000; Bovolenta et al., 2008). Importantly, Crescent and Frzb were recently found to greatly enhance the diffusion of Wnt in *Xenopus* embryos, transporting Wnts and allowing them to signal at considerable distances from where they are secreted (Mii and Taira, 2009).
Perhaps the most surprising function of any sFRP is that of the ventrally expressed sFRP Sizzled (Salic et al., 1997), also called Ogon/Mercedes in zebrafish (Hammerschmidt et al., 1996). Sizzled appears to have lost the Wnt inhibitory activity of its Frizzled domain (Collavin and Kirschner, 2003; Yabe et al., 2003). Importantly, Sizzled acts as a feedback inhibitor of BMP signaling by binding to and competitively inhibiting Tolloids, the metalloproteinases that cleave Chordin (Lee et al., 2006; Muraoka et al., 2006). Sizzled is a key player in D-V self-regulation: when BMP levels increase, sizzled expression in the ventral center increases, causing inhibition of Tolloid enzymes, preventing the release of BMP from Chordin/BMP complexes and, in this indirect way, decreasing BMP signaling (Lee et al., 2006).

Crescent is the closest relative of Sizzled, and was initially isolated in our laboratory as a cDNA expressed in the anterior endomesodermal crescent of the chick embryo (Pfeffer et al., 1997). In Xenopus, Crescent is expressed on the dorsal side of the gastrula in the deep anterior endoderm and later in the prechordal plate (Pera and De Robertis, 2000; Shibata et al., 2000). Crescent differs from Sizzled in that it is able to bind and inhibit Wnt8 and Wnt11 activity in Xenopus embryos (Shibata et al., 2005; Marvin et al., 2001; Schneider and Mercola, 2001; Dickinson and Sive, 2009).

An interesting feature of the D-V patterning pathway is that many of its components have counterparts of similar structure and biochemical activity in the dorsal and in the ventral center. For example, Chordin and CV2, as well as ADMP/BMP2 and BMP4/BMP7, are expressed on opposite sides of the gastrula embryo (Fig. 2-1A). Given the sequence similarity between Crescent and Sizzled, it seemed possible that these two sFRPs could constitute an additional pair of secreted molecules with similar functions, expressed at different poles of the embryo under
opposite transcriptional control. If so, Crescent and Sizzled could provide a new layer of resilience to the D-V patterning pathway.

In this paper we show that Crescent inhibits the activity of Tolloid proteinases and is under the opposite transcriptional regulation from that of Sizzled. Crescent bound to the Tolloid enzyme BMP1 with affinities within the physiological range. Enzyme kinetic analyses showed that Crescent inhibited the cleavage of a fluorogenic peptide substrate mimicking the Chordin cleavage site. Since Crescent itself was not cleaved, it acts as a competitive inhibitor of Tolloid proteinases. This novel function of Crescent in the Chordin/BMP pathway was supported by embryological experiments in which the anti-BMP phenotypes caused by Crescent overexpression were shown to require Chordin. Co-injections of Crescent and Chordin protein into the blastula cavity had synergistic dorsalizing effects. A point mutation mimicking the zebrafish ogon mutation eliminated Tolloid inhibition in biochemical assays and greatly reduced the anti-BMP effects of Crescent protein in Xenopus embryos. We propose that Crescent is a competitive inhibitor of Tolloid proteinases, and a novel component of the extracellular Chordin/BMP biochemical pathway that regulates D-V patterning.
RESULTS

Crescent regulates BMP signaling

The *Xenopus* gastrula contains a dorsal and a ventral signaling center under opposite transcriptional regulation by BMP signaling (Fig. 2-1A). From a molecular standpoint, Crescent resembles Sizzled, Chordin is like CV2, and ADMP/BMP2 and BMP4/BMP7 are all BMPs. Within the sFRP family, Crescent is most similar to Sizzled (Fig. 2-1B). This prompted us to investigate whether Crescent had a similar biochemical activity to that of Sizzled (Lee et al., 2006).

To determine whether the expression of Crescent on the dorsal region of the embryo was transcriptionally repressed by BMP signals, we microinjected BMP4 or Chordin proteins into the blastocoele of stage 8.5 *Xenopus* blastulae (Fig. 2-1C-E). Embryos injected with the BMP antagonist Chordin showed increased levels of *crescent* transcripts (n=9/9) (Fig. 2-1C), while embryos injected with BMP4 showed decreased transcripts (n=11/14) (Fig. 2-1E) when compared to uninjected controls (n=9/10) (Fig. 2-1D) at late gastrula. This indicated that Crescent is normally repressed by BMPs, explaining its preferential expression on the dorsal side of the embryo, where BMP signaling is low.

To study the function of Crescent during gastrulation, an antisense morpholino (Cres MO) was designed. Crescent mRNA or Crescent MO were injected into each blastomere of 4-cell stage *Xenopus* embryos. Sizzled expression was used as the readout for regions of high BMP
Fig. 2-1. *Xenopus crescent* is expressed dorsally and repressed by BMP signaling. 
(A) D-V Patterning is regulated by proteins secreted by the dorsal and ventral signaling centers. For the proteins listed, proteins of similar function are secreted by the two sides, but under opposite transcriptional control.
(B) sFRPs of *Xenopus* (x), human (h), zebrafish (z) and chicken (ch) origin were compared using Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al., 2007). Crescent and Sizzled are philogenetically related, and distant from the other sFRPs. (C-E) *Crescent* expression is under negative transcriptional control by BMP4 signaling. Microinjection of Chordin (Chd) protein increases *crescent* transcripts, while microinjection of BMP4 protein decreases *crescent* expression in stage 12 gastrulae. (F-H) *sizzled* expression is inhibited by injection of *crescent* mRNA and markedly expanded upon depletion of Crescent (Cres MO); an uninjected sibling at stage 11 is shown for comparison.
signaling, since its transcription is activated by BMPs (Collavin and Kirschner, 2003). Embryos overexpressing Crescent showed reduced sizzled expression in the ventral side (n=14/16) (Fig. 2-1F), while depletion of Crescent with MO expanded the ventral sizzled expression (n=9/10) (Fig 1H) compared to uninjected controls (n=10/10) (Fig. 2-1G). Moreover, Crescent overexpression increased Chordin transcripts (Fig. 2-2A, 2-2B), which are normally repressed by BMP signaling (n=27/30). These results indicated that Crescent regulates the BMP signaling gradient and came as a surprise, since Crescent had previously only been implicated in inhibition of Wnt signals (Pera and De Robertis, 2000; Shibata et al., 2005).

To assess the specificity of the Crescent MO, we designed a mutant crescent mRNA, crescentWobble, which contains several mismatches with the antisense morpholino but retains the identical amino acid coding sequence (Fig. 2-2C).

Depletion of Crescent (Cres MO) greatly increased sizzled transcripts (Fig. 2-2E), while overexpression of crescentWobble mRNA reduced them (Fig. 2-2F) compared to control embryos (Fig. 2-2D). Co-injection of Cres MO and crescentWobble mRNA rescued the Crescent MO phenotype (compare Fig. 2-2D to 2-2G), indicating that the phenotype of Cres MO was specific. To further evaluate the ventralizing phenotype displayed upon knockdown of Crescent, total mRNA was collected from embryos injected with Cres MO and uninjected controls, and quantitative RT-PCR were performed. Knockdown of Crescent (Cres MO) led to an increase in the BMP induced genes Xolloid related (Xlr) (Fig. 2-2H) (Dale et al., 2002), BMP4 (Fig. 2-2I), and Vent-1 (Fig. 2-2J), and to a decrease in the BMP repressed gene Chordin (Fig. 2-2K) (Reversade and De Robertis, 2005).

The results presented so far indicate that Crescent normally reduces BMP signaling. Our hypothesis is that Crescent and Sizzled might control Tolloid activity from opposite poles of the
Fig. 2.2. Crescent regulates BMP signaling.

(A,B) Microinjection of crescent mRNA expands the expression of chordin, a gene that is negatively regulated by BMP signaling.

(C) Diagram showing a control crescent mRNA containing wobble position mutations is no longer targeted by Crescent MO.
(D-G) Crescent MO greatly expands expression of the ventral (high-BMP) marker sizzled, while crescent Wobble mRNA reduces Sizzled expression and rescues the effects of Crescent MO. (H-K) Knockdown of Crescent increases the transcript levels of BMP induced genes (Xlr, BMP4, Vent-1), while reducing the levels of chordin, a gene repressed by BMP signaling. (L-O) chordin mRNA expression is reduced by Crescent MO or Sizzled MO. Note that simultaneous depletion of both sFRPs causes a synergistic ventralizing effect, greatly reducing chordin transcripts at early gastrula. (P) Model in which the BMP gradient is represented by a see-saw in which dorsal and ventral inhibitors of Tolloid metalloproteinases adjust the D-V gradient through the proteolytic degradation of Chordin. Blue arrows symbolize transcriptional regulation by BMPs, black arrows indicate direct protein-protein interactions.
embryo. When Crescent is depleted, increased expression of Sizzled may compensate in part for the lack of Crescent. If this were the case, simultaneous depletion of both Crescent and Sizzled should better relieve Tolloid proteinases from inhibition and cause increased ventralization of the gastrula. Chordin expression was used to probe for BMP signaling levels, since Chordin is transcriptionally inhibited by BMP signals (Reversade and De Robertis, 2005). Embryos depleted of either Crescent (n=24/28) or Sizzled (32/35) showed reduced chordin transcripts (Fig. 2-2I-K). Indeed, embryos doubly depleted of Crescent and Sizzled showed even more severely reduced chordin levels (n=29/31) (Fig. 2-2L). We propose that Crescent and Sizzled cooperate in vivo, from opposite poles of the embryo, to fine tune the D-V BMP signaling gradient during development (Fig. 2-2P).

**Crescent inhibits the degradation of Chordin by Tolloid metalloproteinases**

We next asked whether the mechanism by which Crescent reduced BMP signaling was through inhibition of the Tolloid proteinases that cleave the BMP antagonist Chordin (Fig. 2-2M). To test this hypothesis, we conducted biochemical studies measuring the digestion of Chordin by Tolloid in the presence of Crescent (Fig. 2-3A, B). The reactions were performed using affinity-purified Crescent protein (Cres-Flag or Cres-HA) as the inhibitor, Xenopus Chordin (Chd-myc) produced in baculovirus as the substrate, and either commercial hBMP1 or affinity-purified Xolloid-related-Flag (Xlr) as the proteinase. The digestion reactions were performed as described in Piccolo et al. (1997), and analyzed by Western blots using antibodies against the Chd-Myc tag.
Fig. 2. Crescent inhibits the proteolytic activity of Xlr or BMP1 on Chordin.

(A) Purified Crescent-HA (150 nM) inhibited the proteolysis of Chordin-myc by Xlr to the same extent as Sizzled-Fc at the same concentration.

(B) Crescent-HA inhibited in a dose-dependent manner (16, 50, 150 nM) the cleavage of Chordin (30 nM) by BMP1 (10 nM).

(C) Crescent-Flag (150 nM) was not cleaved by BMP1 (10 nM) (compare lane 1 and 2), while Chordin-myc (30 nM) was cleaved in the same experiment (lanes 3 and 4).

(D) Crescent proteins affinity-purified over an anti-flag matrix stained with Coomassie Brilliant Blue to indicate their purity.
Chd-myc was digested by purified Xlr protein (Fig. 2-3A, compare lanes 1 and 2). Addition of Crescent protein inhibited the proteolytic cleavage of Chordin by Xlr (compare lanes 2 and 3); the level of inhibition was comparable to that achieved by the same concentration of Sizzled (Fig. 2-3A, lane 4), a bona fide Tolloid inhibitor (Lee et al., 2006). This result could also be reproduced using another Tolloid proteinase, hBMP1 (data not shown). Furthermore, the inhibitory effect of Crescent on Tolloid enzymes was dose-dependent (Fig. 2-3B).

One possible explanation for the inhibition of Tolloid activity could be that Crescent itself might be a substrate for Tolloid proteinases. This possibility could be ruled out, for Crescent-Flag protein was not digested by BMP1 (Fig. 2-3C, lanes 1 and 2), while Chordin was degraded in the same experiment (Fig. 2-3C, lanes 3 and 4). The purity of our Crescent-flag proteins was verified through Coomassie Brilliant Blue staining (Fig. 2-3D). These results show that Crescent protein is an inhibitor of Tolloid proteinases.

**Crescent binds and competitively inhibits Tolloid proteinases**

To analyze the mechanism by which Crescent inhibits Tolloids, a Chordin-mimicking fluorogenic substrate was used (Chd-peptide). This fluorogenic substrate is an octapeptide containing target amino acids of Chordin recognized by Tolloid metalloproteinases. The octapeptide is flanked by a fluorophore on one side and its quencher on the other (Lee et al., 2009). Upon cleavage by Tolloids, the fluorophore is released from quenching and emits fluorescence at 405 nm. When the kinetics of the enzyme reaction was analyzed at increasing concentrations of the substrate (fluorogenic Chd-peptide), double reciprocal Lineweaver-Burk plots (Fig. 2-4A) showed that Crescent displayed typical competitive inhibitor kinetics (Dixon
Fig. 2-4. Crescent binds and competitively inhibits Tolloid Proteinases.
(A) Lineweaver-Burk plot (1/v_i over 1/[S]) showing that Crescent is a competitive inhibitor of Tolloid proteinases.
(B) Dixon Plot ($1/v_i$ over $[I]$) from which the Ki (59 nM) could be obtained directly.

(C) BIAcore surface Plasmon resonance sensograms for the Crescent-BMP1 interaction in real time, showing an average $K_D$ of 11 nM. The points in time when BMP1 binding starts, and when washing with buffer starts, are indicated.
and Webb, 1979): at two concentrations of Crescent-Flag, the apparent Km for the substrate changed, while the maximal velocity ($V_{max}$) did not.

The inhibition constant ($K_i$) is defined as the concentration of inhibitor at which half of the enzyme is in complex with the inhibitor. The $K_i$ for Crescent inhibition was obtained using a Dixon Plot (Fig. 2-4B), a graphical method which yields the $K_i$ directly without calculation. The initial velocities ($v_i$) were determined at a series of inhibitor concentrations, and then plotted as $1/v_i$ against the concentration of inhibitor [I]. These reactions were performed at two substrate concentrations, producing two slopes. The point at which both these lines intersect is equal to -$K_i$ in the case of a competitive inhibitor (Dixon and Webb, 1979). The $K_i$ of Crescent determined by this method was 59 nM (Fig. 2-4B).

After determining that Crescent is a competitive inhibitor of Tolloids, we analyzed the physical properties of their interaction. The binding affinity between Crescent and Tolloid was measured by surface plasmon resonance (BIACore) analyses (Fig. 2-4C). Crescent-Flag proteins were covalently crosslinked to the surface of a sensor chip. hBMP1 was passed over this chip at constant flow, and changes in the refractive index caused by associations and dissociations recorded for different concentrations of hBMP1 (Fig. 2-4C). The affinity of the binding, expressed by the dissociation constant ($K_D$), was calculated from the quotient between the kinetic rates of association and dissociation. The $K_D$ for the interaction between Crescent and BMP1 was on average 11 nM, which corresponds to a binding of high affinity within the physiological range (Hojima et al., 1985).

We conclude from these biochemical studies that Crescent competes for the binding of Chordin substrates to the BMP1/Tolloid catalytic site, and that this binding has a dissociation constant in the $10^{-9}$ Molar range.
**Chordin is required for the dorsalizing activity of Crescent**

In previous work, the clue that led to the discovery of the mechanism of action of Sizzled was the observation that microinjections of *sizzled* mRNA had no effect on ventral half-embryos bisected at blastula. This indicated that Sizzled required a dorsal component in order to mediate its dorsalizing (anti-BMP) effects (Lee et al., 2006). A similar approach was taken here for Crescent. Microinjections of Crescent mRNA dorsalized whole *Xenopus* embryos, as indicated by the expansion of the pan-neural marker *SOX2* (Fig. 2-5A, B). To determine whether dorsal components were required for this anti-BMP activity, bisection experiments were carried out (Revorsade and De Robertis, 2005). Only the dorsal halves of Crescent-injected embryos showed an expansion of *SOX2* (n=16/16), while ventral halves were unaffected by Crescent overexpression (Fig. 2-5B). Control experiments with *chordin* mRNA showed that this BMP inhibitor very effectively dorsalized ventral half-embryos (Oelgeschläger et al., 2003 and data not shown). These results show that Crescent is incapable of affecting ventral tissues directly, and requires dorsal components in order to elicit its effects.

Our biochemical experiments indicated that Chordin was the likely dorsal component required for the dorsalizing activity of Crescent. To test this hypothesis, we injected Crescent protein into blastula embryos that had been previously depleted of Chordin by antisense Chd morpholino oligos (Oelgeschläger et al., 2003). As expected, Chordin-depleted embryos showed classical ventralized features (n=15/16), with reduction of *Rx2a* and an increase of posterior *Sizzled (Szl)*, which marks ventral BMP4/7 signaling (Fig. 2-5C, D). Crescent protein injected into the blastocele caused dorsalization with an expansion of the forebrain and eye marker *Rx2a*.
Fig. 2-5. Crescent requires Chordin in order to dorsalize the embryo.
(A) Embryos bisected along their D-V axis. The dorsal half self-regulates, forming a well-proportioned embryo, while the ventral half forms a belly piece consisting of ventral tissues.
(B) Crescent mRNA microinjection increases SOX2 expression in dorsal halves, but has no effect on ventral half-embryos. Thus, the dorsalizing activity of Crescent requires a dorsal component.
(C) Uninjected control embryos showing normal Rx2a and Sizzled transcript levels at stage 20.
(D) Embryos injected with Chordin MO showing a ventralized phenotype consisting of reduced Rx2a and expanded posterior Sizzled transcripts (inset).
(E) Injection of Crescent protein into the blastocoele dorsalizes embryos, expanding \textit{Rx2a} expression, decreasing \textit{Sizzled} expression in the posterior-ventral region and increasing \textit{Sizzled} in the anterior-ventral region (where BMP2 is expressed).

(F) Injections of Crescent protein into Chordin-depleted embryos are without dorsalizing effects; this result indicates that Crescent protein requires Chordin to dorsalize \textit{Xenopus} embryos. Insets show lateral views.
(n=36/39) (Fig. 2-5E, compare to 2-5C). (The anterior and ventral expression of Sizzled also observed in 5E is due to the upregulation of BMP2, in dorsalized embryos; BMP2 is expressed in this region when BMP signaling is lowered, Inomata et al., 2008). However, in embryos that had been previously depleted of Chordin, Crescent protein was devoid of dorsalizing (anti-BMP) activity, as indicated by the reduction of Rx2a and residual posterior Szl expression (n=29/34) (Fig. 2-5F). These results indicate that Crescent requires Chordin in order to dorsalize the Xenopus embryo.

A Crescent mutant mimicking an Ogon mutation has impaired anti-BMP activity

The isolation of the ogon/mercedes mutation in zebrafish opened the way to the discovery of the role of Sizzled in BMP signaling. This point mutation is located in the cysteine-rich frizzled domain. SizzledSGON, in which an Aspartic acid is replaced by an Asparagine, renders Ogon/Sizzled biologically inactive (Yabe et al., 2003). Introducing this mutation into Xenopus sizzled (sizzledSGON) causes it to lose its ability to inhibit Tolloid Proteinases (Lee et al., 2006). Taking advantage of the sequence conservation between Crescent and Sizzled, we made a construct mimicking the ogon mutation in Crescent, CrescentDG103N (Fig. 2-6A). When microinjected into the blastocele of Xenopus embryos, affinity-purified CrescentDG103N protein (n=18/21) was unable to expand the dorsal marker Rx2a when compared to the same amount of microinjected CrescentWT protein (18/18) (Fig. 2-6B-D). In biochemical experiments, CrescentWT inhibited cleavage of the fluorogenic Chd-peptide by BMP1 enzyme in a dose-dependent manner (Fig. 2-6E), while the point mutant CrescentDG103N failed to inhibit the reaction at the same concentrations (Fig. 2-6F). Moreover, embryos injected with CrescentDG103N were less
Fig. 2-6. A Crescent mutant mimicking the Ogon mutation lacks Tolloid inhibitory activity and has less anti-BMP activity in the Xenopus embryo.

(A) Flag-tagged affinity-purified protein Crescent\textsuperscript{WT} and Crescent\textsuperscript{D103N}.

(B-D) Microinjection of Crescent\textsuperscript{WT} protein (2.5 μM) into the blastocele dorsalizes embryos and expands \textit{Rx}2\textit{a} expression, while microinjection of the same concentration of Crescent\textsuperscript{D103N} had reduced dorsalizing ability and was unable to expand \textit{Rx}2\textit{a} expression.
(E, F) Crescent\textsuperscript{WT} inhibited cleavage of a fluorogenic Chordin peptide by BMP1 enzyme in a dose-dependent manner, whereas Crescent\textsuperscript{D103N} was unable to inhibit this reaction. (G-I) Crescent\textsuperscript{D103N} is able to inhibit the induction of secondary axes by \textit{xWnt8} mRNA, indicating that the Wnt-inhibiting and Tolloid-inhibiting activities of Crescent\textsuperscript{WT} are separable. (J-L) A sub-threshold amount of Chordin protein injected into the blastocoele has very limited effect. If, in addition to this amount of Chordin, embryos also received a modest amount of Crescent\textsuperscript{WT}, synergetic cooperation between Crescent and Chordin proteins was observed, manifested as an extreme increase in \textit{Rx2a} expression in ectoderm. Embryos injected with Crescent\textsuperscript{D103N}, although retaining a dorsally “kinked” phenotype (probably caused by inhibition of convergence and extension movements that require Wnt signaling), did not exhibit this increase in the \textit{Rx2a} forebrain marker, when co-injected with Chordin protein. Insets show frontal views of embryos without injection of Chordin protein.
dorsalized than those injected with Crescent\textsuperscript{WT} protein (Supplemental Fig. 2-S1). The Crescent\textsuperscript{D103N} mutant loses Tolloid-inhibiting activity, but retains its ability to inhibit Wnt signaling. This was ascertained by coinjecting Wnt8 mRNA together with crescent\textsuperscript{WT} or crescent\textsuperscript{D103N} mRNA (Fig. 2-6G-I). Axis induction by xWnt8 mRNA (n=39/47, 83% with secondary axes) was blocked both by crescent\textsuperscript{WT} (n=55/55) or crescent\textsuperscript{D103N} (n=52/52). We note that at 5 pg of xWnt8 mRNA, although secondary axes were eliminated, some dorsalizing activity persisted (although at 2.5 pg all effects of xWnt8 were eliminated), as shown in Fig. 2-6I. This indicates that a small amount of anti-Wnt potency might be affected in the mutant. We conclude that the two functions of Crescent are separable, with Crescent\textsuperscript{D103N} losing its Tolloid-inhibiting activity but retaining its function as a Wnt inhibitor. The dorsalizing phenotypic activity of Crescent can be mostly ascribed to its effects on BMP signaling through Tolloid inhibition.

We next tested the effect of Crescent and its Ogon-like mutant in embryos sensitized by microinjection of a low amount of Chordin protein that does not change D-V patterning on its own (Fig. 2-6J, compare with its inset). Crescent\textsuperscript{WT} protein was injected into the blastocoel in amounts that only slightly increased dorsalization (n=8/8) (Fig. 2-6J, inset). Interestingly, when Chordin and Crescent proteins were co-injected, a massive expansion of the forebrain marker Rx2a was induced (n=9/12) (Fig. 2-6K). This strong synergy between Chordin and Crescent on Rx2a expression was not observed when the same amount of Crescent\textsuperscript{D103N} protein was co-injected (n=6/6) (compare Fig. 2-6K to 2-6L). The equivalent point mutations in the frizzled domain of Crescent (this work) or of Sizzled (Lee et al., 2006) caused both proteins to lose their metalloproteinase inhibiting activity. The remarkable synergistic interaction between
microinjected Chordin and Crescent is explained by the inhibition of endogenous Tolloid enzymes, which normally degrade Chordin, by Crescent protein.

**DISCUSSION**

**Crescent is a Tolloid Inhibitor**

An intriguing question is why certain putative Wnt antagonists bearing frizzled domains, such as Crescent or Sizzled, and BMP antagonists such as Chordin or Noggin, cause very similar overexpression phenotypes. For example, in zebrafish overexpression of Sizzled or Chordin causes embryos to become dorso-anteriorized and lose ventral BMP-dependent structures such as the ventral fin (Yabe et al., 2003). This puzzle started to be answered when the surprising molecular mechanism of Sizzled action was discovered. Sizzled plays a critical role in the BMP pathway as a competitive inhibitor of the Tolloid proteinases that degrade the BMP antagonist Chordin, rather than through the modulation of Wnt signaling (Lee et al., 2006, Muraoka et al., 2006).

This unexpected function of Sizzled in Chordin proteolysis led us to test here whether the sFRP Crescent, expressed in the opposite pole of the developing gastrula, might have a similar function. A few hints pointed in this direction: within the sFRP family, Crescent is closest to Sizzled in terms of primary structure (44% identity, 63% similarity) (Collavin and Kirschner, 2003), and is expressed at the time and place in the developing gastrula at which Tolloid inhibition is likely to be important (Pera and De Robertis, 2000; Shibata et al., 2000). Crescent has well-documented effects on Wnt8 and Wnt11 activity in *Xenopus* embryos (Shibata et al.,
Crescent inhibits Tolloid proteinases, which are known to provide the rate-limiting step in the Chordin/BMP pathway (Lee et al., 2009). Using biochemical and embryological approaches, we found that Crescent is a competitive inhibitor of toloids, which enhance BMP signaling by inhibiting the cleavage of the BMP antagonist Chordin in the extracellular space. Crescent protein inhibited the proteolytic cleavage of Chordin by Xlr or BMP1 in vitro, without itself being cleaved by the proteases. Crescent functions as a competitive inhibitor of Tolloid metallopeproteinases. We also found that Crescent protein was able to bind BMP1 with high affinity, in the physiologically relevant nanomolar range (Hojima et al. 1985; Lee et al., 2006). Tolloid inhibition explains the dorsalized phenotypes caused by Crescent protein microinjections. Crescent was unable to dorsalize ventral half-embryos and required endogenous Chordin to dorsalize intact Xenopus embryos. Crescent\(^{D103N}\) is a point mutant mimicking the Ogon mutation in Sizzled, which loses its Tolloid inhibiting activity in vitro while retaining its Wnt-inhibiting properties. Interestingly, this mutant displayed a much reduced dorsalizing capacity and was ineffective in cooperating with Chordin protein when coinjected into Xenopus embryos (Fig. 2-6). Thus, the Tolloid inhibiting activity of Crescent appears to be responsible for a significant part of its phenotypic effects in Xenopus embryos.
Crescent is at the intersection of Wnt and BMP signaling

*Xenopus* Crescent was isolated in a screen for proteins secreted in gastrulating *Xenopus* embryos and was found to be expressed in Spemann’s organizer, in particular at the leading edge of the endomesoderm (Pera and De Robertis, 2000). Like all sFRPs, Crescent contains a cysteine-rich domain (CRD) homologous to the extracellular domain present in Frizzled receptors (Pera and De Robertis, 2000; Shibata et al., 2000). This is why most sFRPs, with the exception of Sizzled, are thought to bind Wnt ligands and regulate Wnt signaling (Leyns et al., 1997; De Robertis and Kuroda, 2008, Bovolenta et al., 2008).

Crescent binds to with Wnt11 and Wnt5a and has a role in the regulation of convergent extension movements during gastrulation and neurulation, via modulation of the non-canonical Wnt pathway (Pera et al., 2000; Shibata et al., 2005). The role of Crescent as a Wnt inhibitor is displayed during the development of the stomodeum or mouth, in which Crescent and Frzb are required to antagonize Wnt signaling, low levels of Wnt are essential for perforation of the buccopharyngeal membrane (Dickinson and Sive, 2009). Wnt signaling inhibition by Crescent is also critical for heart development (Marvin et al., 2001; Schneider and Mercola, 2001).

Recently, a new twist in the Wnt-regulating ability of Crescent has been discovered. Experiments in *Xenopus* have demonstrated that Crescent and Frzb enhance the diffusion of Wnts in the gastrulating embryo (Mii and Taira, 2009). Crescent increased the diffusion of Venus-tagged Wnt8 and Wnt11. Crescent is thought to achieve this by binding and transporting Wnts, improving their diffusibility. Wnt ligands transported by Crescent retain signaling capacity and Crescent has a biphasic effect: at low concentrations the interaction between Crescent and Wnt8 causes an increase in canonical Wnt signaling at a distance, while at high concentrations
Crescent acts as a Wnt antagonist. It is conceivable that the increased diffusibility of Wnt ligands when co-injected with Crescent might be related to the inhibition of Tolloid metalloproteinases. Tolloid proteinases digest a diverse set of precursors into mature functional proteins. Among these substrates are many proteins important in the formation of the extracellular matrix, such as fibrillar procollagens (BMP1 is also known as Procollagen C peptidase), Latent TGF-β-binding protein (LTBP), Lysyl oxidase, Promyostatin, Osteoglycin and Biglycan (Hopkins et al., 2007). Tolloids play fundamental roles in development, and it is thought that many of these involve remodeling of the extracellular matrix (Hopkins et al., 2007). Inhibition of Tolloids, causing failure to properly process precursors of the extracellular matrix, might modify the landscape through which morphogens need to diffuse.

One important question that we were unable to address experimentally is whether the binding of Wnt to Crescent modified its ability to inhibit Tolloid protease activity. This experiment was attempted, but failed because the detergent-containing buffer required to maintain purified Wnt in solution, as well as conditioned medium from cell cultures, are incompatible with the biochemical assays of Tolloid activity. It is, however, an interesting question to contemplate, for if Wnt binding to Crescent modulated its inhibition of Tolloid, Crescent might behave as a receptor of a Wnt activity that would function entirely in the extracellular space.

sFRP constitute the largest family of Wnt inhibitors, for which a growing number of novel Wnt-independent functions are being discovered (Bovolenta et al., 2008). Of these alternative Wnt-independent roles for sFRPs, the best established one is that of Sizzled as a competitive inhibitor of Tolloid Proteinases (Lee et al., 2006; Muraoka et al., 2006). Sizzled does not antagonize Wnt signaling in vivo (Collavin and Kirschner, 2003; Yabe et al., 2003), but
instead acts as a feedback inhibitor of BMP signaling by competitively inhibiting the cleavage of the BMP antagonist Chordin (Lee et al., 2006; Muraoka et al., 2006). In addition, sFRP2 has been recently shown to act as an enhancer of the procollagen C proteinase activity (Kobayashi et al., 2009). It seems likely that additional functions for sFRPs will continue to be uncovered.

**Crescent is part of the D-V patterning pathway**

As shown in Fig. 2-7, patterning of the D-V axis is mediated by an extracellular network of interacting proteins. At the heart of this network is the signaling gradient generated by BMP-Chordin antagonism (Fig. 2-7A). BMPs (BMP2/4/7 and ADMP) are secreted both ventrally and dorsally, and their signaling causes cell differentiation towards ventral fates. The dorsally-secreted antagonist Chordin binds BMPs and impedes the binding of BMPs to their receptors, ensuring that the dorsal side of the embryo is kept free of BMP signaling (Piccolo et al., 1996). The Chordin/BMP antagonism creates a gradient of BMP signaling along the D-V axis, which is reinforced by an array of proteins that help keep BMP signaling high on the ventral and low in the dorsal side, either by sequestering, transporting or solubilizing BMP ligands (Zakin and De Robertis, 2010).

Many components of this extracellular network that regulates D-V patterning have counterparts of similar structure and function secreted under opposite transcriptional control on the other side of the embryo. Examples of this are BMP2 and ADMP on the dorsal and BMP4 and BMP7 on the ventral side (Reversade and De Robertis, 2005; Inomata et al., 2008), as well as Chordin dorsally and the structurally related protein CV2 on the ventral side (Ambrosio et
Fig. 2-7. Model of the extracellular network of proteins that control D-V patterning. Crescent was shown here to be a component of the extracellular biochemical network that controls D-V patterning. Arrows in blue represent transcriptional regulation by BMPs, arrows in black symbolize direct protein-protein interactions, and the red arrows indicate flux of Chordin/ADMP/BMP from the dorsal toward the ventral side of the embryo. Each protein-protein interaction indicated here is supported by biochemical and embryological studies in *Xenopus*.

(A) The BMP/Chordin gradient plays a central role in D-V patterning.
(B) Tolloid metalloproteinases are inhibited by Crescent from the dorsal and Sizzled from the ventral side.
(C) The extracellular biochemical pathway of D-V development in *Xenopus* embryos, including the flux of Chordin/BMP complexes from dorsal to ventral. Two important regulators of the Chordin/BMP pathway have been omitted for simplicity in this diagram. One is Ont-1, an adaptor Olfactomedin-related protein that binds both Chordin and Tolloid, which facilitates Chordin proteolysis (Inomata et al., 2008). The other regulator is Twisted-gastrulation (Tsg), a protein that binds both BMP and Chordin, facilitating BMP signaling, the binding of BMP to Chordin, and the cleavage of Chordin by Tolloids (De Robertis and Kuroda, 2004; Little and Mullins, 2006).
The experiments presented here indicate that Crescent and Sizzled constitute a new pair of proteins expressed at opposite poles of the embryo and having similar functions (Fig. 2-7B). Their opposing transcriptional regulation should confer additional resilience and robustness to the regulatory network. For example, in loss of function experiments when Crescent expression is knocked down by morpholinos, the transcriptional upregulation of Sizzled is able to compensate in part, explaining why the double knockdown of Crescent and Sizzled causes a much severe high-BMP phenotype (Fig. 2-2).

BMP antagonism by Chordin is not irreversible. BMPs can be liberated from this inhibition by Tolloid metalloproteinases that cleave Chordin, enabling BMPs to signal again (Piccolo et al., 1997). In the *Xenopus* embryo, 3 Tolloid enzymes are expressed: BMP1, Xolloid (Xld) and Xolloid Related (Xlr). While the first two are expressed ubiquitously in the early embryo, Xlr is transcriptionally upregulated in more ventral regions by BMP4/7 signaling (Goodman et al., 1998; Dale et al., 2002). These metalloproteinases release BMPs from the Chordin-BMP complex, allowing BMPs to signal (Fig. 2-7C). The regulation of Tolloid activity is crucial to D-V patterning.

CV2 is a secreted molecule that contains Chordin-like domains that bind BMPs, but remains localized at its site of synthesis on the ventral side (Rentzsch et al., 2006, Serpe et al., 2008; Ambrosio et al., 2008; Zakin et al., 2010). Acting as an anti-BMP, CV2 binds BMPs and promotes their endocytosis and destruction, serving as a feedback inhibitor (Zhang et al., 2008; Kelley et al., 2009). CV2 also acts as a pro-BMP. Since it remains near the cells where it was produced, CV2 also functions as a molecular sink, concentrating Chordin/BMP complexes diffusing from more dorsal regions on the ventral side (Ambrosio et al., 2008; Blair, 2007). Once concentrated on the ventral region, Chordin/BMP complexes can be cleaved by tolloid(s) (with the
help of the extracellular Ont-1 scaffold protein, Inomata et al., 2008), liberating active BMPs (Fig. 2-7C) which allow the embryo to reach peak BMP signaling levels in the ventral center (reviewed in Zakin and De Robertis, 2010).

Tolloid metalloproteinases play a crucial role in the D-V patterning pathway, and the ventral and dorsal signaling centers regulate their proteolytic activity by secreting proteins that can inhibit these enzymes. An additional inhibitory feedback loop is provided by the recently discovered ability of BMP4 to inhibit Tolloids (Fig. 2-7C) when its levels become high (Lee et al., 2009). Since Tolloid proteinases constitute the rate-limiting step in the D-V Chordin/BMP pathway, it is not surprising that these enzymes are highly regulated. Sizzled, produced abundantly on the ventral side (Lee et al., 2006), serves as an important negative feedback inhibitor. On the opposite side of the gastrulating embryo, Crescent similarly protects Chordin from Tolloid metalloproteinases by competitively inhibiting them, ensuring optimal levels of Chordin on the dorsal side. Self-regulation of D-V pattern results from this opposition between dorsal and ventral secreted BMP signals and their extracellular regulators.

**Crescent in evolution**

From an evolutionary standpoint, the Chordin/BMP/Tolloid pathway represents the ancestral D-V patterning system, which has been conserved in the early embryos of *Drosophila*, beetles, spiders, hemichordates, amphioxus, fish, amphibians and birds (reviewed in De Robertis, 2008; Umulis et al., 2009). *Drosophila* has retained many components of the system (Short-gastrulation/Chordin, Dpp/BMP, Tolloid, Tsg and CV2), but its genome does not contain even a single sFRP. Therefore, the D-V system of secreted proteins can generate pattern without
Crescent or Sizzled. However, sFRPs are found in some invertebrates such as nematodes and annelids, so insects must have undergone gene loss. Like *Xenopus*, the chick embryo expresses Crescent and Sizzled abundantly in dorsal and ventral regions, respectively (Pfeffer et al., 1997; Wittler et al., 2008). It is therefore very surprising that a functional Crescent is not present in the genome of placental mammals. The platypus (*Ornithorhynchus anatinus*), contains a perfect copy of Crescent (Warren et al., 2008). An intact *crescent* gene is also found in the opossum (*Monodelphis domestica*), a marsupial. However, in the genome of placental mammals Crescent can no longer be identified, except in the dog genome (*Canis lupus familiaris*) (Lindblad-Toh et al., 2005), in which a pseudogene containing multiple inactivating mutations and deletions in Crescent is still recognizable (J.L. Plouhinec and E.M.D.R., unpublished observations). Perhaps the fine regulation of D-V pattern was no longer needed after the mammalian egg lost its yolk (which is still present in the platypus) and consequently the need for self-regulation of pattern during the epiboly movements by which the embryo surrounds the yolk. In evolution, once a gene is no longer needed, it is rapidly lost (De Robertis, 2008). Although no longer required for patterning mice and men, Crescent is a conserved gene important in the regulative development of lower vertebrates, which functions in both the Wnt and the BMP signaling pathways.
MATERIALS AND METHODS

Morpholino Oligos and Embryonic Manipulations

Antisense Morpholinos (Gene Tools) were as described: Chordin MO (Oelgeschläger et al., 2003), Sizzled MO (Collavin and Kirschner, 2003), and Crescent MO (5’-CTCTGACACACCTGAGGGCCATT-3’). Each MO was microinjected four times radially into 4-cell embryos (34 ng total). Bisection experiments were performed by cutting stage 9 embryos across their prospective D–V axis into two equal halves, using a surgical blade. Embryos were bisected and cultured in 0.3 x Modified Barth solution (Gurdon, 1976; Reversade and De Robertis, 2005). For mRNA microinjection, 200 pg of *Xenopus crescent* (Pera and De Robertis, 2000) or *crescent*Wobble were injected four times radially into 2- or 4-cell embryos. *Crescent*Wobble (…5’ ATG GCT CCA CAA CTG TGC CAA 3’…) was generated by introducing 5 synonymous mutations (underlined nucleotides) in the wild type *Xenopus laevis* Crescent sequence targeted by our Crescent Morpholino. Double axes were induced by microinjections of 2.5 or 5 pg of Wnt8 mRNA and inhibited with 200 pg of Crescent or CrescentD103N mRNA. For protein microinjections, affinity-purified Crescent-Flag or CrescentD103N-Flag (both at 5 µM, 60 nl), and recombinant mouse Chordin (2.5 µM, 60 nl) or human BMP4 (0.4 µM, 60nl, R&D Systems) were microinjected into the blastocoelle at mid-blastula (stage 8.5). Detailed procedures for whole-mount in situ hybridization are available at http://www.hhmi.ucla.edu/derobertis/protocol_page/protocol.html
Biochemical Methods

*Xenopus* Chordin-Myc was produced in baculovirus (Piccolo et al., 1996) and subsequently affinity-purified or used directly as substrate. *Xenopus* Crescent-HA and Crescent-Flag were tagged at the C-terminus by PCR. To generate Crescent\(^{D103N}\), the site-directed mutagenesis Quikchange kit (Stratagene) was used. These proteins, plus Xlr-PC and Szl-Fc (Lee et al. 2006), were produced by transient transfection (Fugene, Roche) of HEK 293T cells. Conditioned medium containing secreted proteins was affinity-purified using PC (Roche), Protein A (Sigma), Flag (Sigma), or HA beads (Covance) according to manufacturer instructions. For BMP1 enzymatic assays, commercial recombinant human BMP1 protein (R&D Systems) and a fluorogenic substrate synthesized based on the sequence of the main cleavage site of Tolloid on Chordin, Mca-SMQSGAK-Dnp (Bachem), were used (Lee et al., 2009). Reactions were performed with 25 μM fluorogenic Chordin-peptide substrate in Xld Buffer (Piccolo et al. 1997) with the addition of 0.01% Brij 35.

Enzymatic activities were measured in a fluorescent plate reader (excitation = 320 nm, emission = 405 nm) and initial velocities calculated from the rate of fluorescence increase in 60 minute reactions. For enzyme kinetics studies, Lineweaver-Burk plots were constructed using initial velocities (\(v_i\)) obtained from fluorometric enzyme assays at different Chd-peptide and Crescent-Flag concentrations. Dixon plots were generated by modifying the concentration of inhibitor for two different substrate concentrations, and plotting the inverse of the initial velocity versus the concentration of inhibitor. The kinetic constants \(K_m\) (Michaelis constant), \(V_{max}\) (maximal velocity) and \(K_i\) (inhibition constant) were calculated as described in Dixon and Webb (1979). For Xlr and BMP1 *in vitro* enzymatic digestion assays, 30 nM baculovirus Chd-Myc was
incubated in Xld Buffer with affinity-purified Xlr-Flag or human BMP1 (R&D Systems) containing the indicated concentrations of Szl-Fc, Crescent-Flag or Crescent-HA at 25°C (or 37° in a few instances) for 2 hr. Western blots were visualized using pico chemiluminescent substrate (Pierce) or the LiCOR Odyssey infrared imager system.

**Surface Plasmon Resonance Analyses**

Surface plasmon resonance measurements were performed in a BIAcore 3000 system. Affinity purified Crescent-Flag protein, diluted to 20 μg/ml in 10 mM sodium acetate (pH 5.0), was bound on a carboxymethylated dextran (CM5) sensor chip using amine coupling to a level of approximately 6000 response units. Binding of recombinant human BMP1 protein (carrier free, R&D Systems) and washes were performed in Xld Buffer. Each experimental cycle consisted of a flow Crescent-Flag at various concentrations. After each cycle, non-crosslinked proteins were removed by a flow of 10 mM HCl in order to regenerate the chip surface. Data were analyzed with BIAevaluation 4.1 software and curve-fitting was done with the assumption of one-to-one binding (Wang et al., 2003).

**Quantitative RT-PCR**

Total RNA from 10 whole embryos (at stage 11) per sample was extracted with the Absolutely RNA Microprep kit (Stratagene). Synthesis of cDNA was done using random hexamer primers and the StrataScript Reverse Transcriptase (Stratagene). Quantitative RT–PCR was performed on the Mx3000P (Stratagene) apparatus using the Brilliant SYBR Green QPCR
Master Mix (Stratagene). Measurements were performed in quadruplicates and normalized to the expression levels of ODC (Ornithine decarboxylase). The formula $2^{-\Delta\Delta Ct}$ was used to calculate fold induction values. Bars indicate standard deviations. RT-PCR conditions and primers can be found at http://www.hhmi.ucla.edu/derobertis/protocol_page/protocol.html.

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Fig. 2-S1. A mutation in Crescent (Crescent$^{D103N}$) deficient in its ability to inhibit tolloid proteinases, also has less dorsalizing capacity in *Xenopus* embryos.  
(A) Microinjections of Crescent$^{WT}$ protein (2.5 μM) into the blastocele cause dorsalization in *Xenopus* embryos.  
(B) Microinjections of Crescent$^{D103N}$ (2.5 μM) had reduced dorsalizing capacity.
Chapter 3

Presenilin Deficiency or Lysosomal Inhibition Enhance Wnt Signaling Through Relocalization of GSK3 to the Late Endosomal Compartment

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Sustained canonical Wnt signaling requires the inhibition of glycogen synthase kinase 3 (GSK3) activity by sequestration of GSK3 inside multivesicular endosomes (MVEs). Here, we show that Wnt signaling is increased by the lysosomal inhibitor chloroquine, which causes accumulation of MVEs. A similar MVE expansion and increased Wnt responsiveness was found in cells deficient in presenilin, a protein associated with Alzheimer's disease. The Wnt-enhancing effects were entirely dependent on the functional endosomal sorting complex required for transport (ESCRT), which is needed for the formation of intraluminal vesicles in MVEs. We suggest that accumulation of late endosomal structures leads to enhanced canonical Wnt signaling through increased Wnt-receptor/GSK3 sequestration. The decrease in GSK3 cytosolic activity stabilized cytoplasmic GSK3 substrates such as β-catenin, the microtubule-associated protein Tau, and other proteins. These results underscore the importance of the endosomal pathway in canonical Wnt signaling and reveal a mechanism for regulation of Wnt signaling by presenilin deficiency.
ABSTRACT

Sustained canonical Wnt signaling requires inhibition of Glycogen Synthase Kinase 3 (GSK3) activity through its sequestration inside multivesicular endosomes (MVEs). Here we show that Wnt signaling is increased by the lysosomal inhibitor Chloroquine, which causes accumulation of MVEs. A similar MVE expansion and increased Wnt responsiveness was found in cells deficient in Presenilin, a protein associated with Alzheimer’s disease. The Wnt-enhancing effects were entirely dependent on functional endosomal sorting complex required for transport (ESCRT), which are needed for formation of intraluminal vesicles in MVEs. We suggest that accumulation of late endosomal structures leads to enhanced canonical Wnt signaling through increased Wnt-receptor/GSK3 sequestration. The decrease in GSK3 cytosolic activity stabilized cytoplasmic GSK3 substrates such as β-Catenin, the microtubule associated protein Tau and other proteins. These results underscore the importance of the endosomal pathway in canonical Wnt signaling and reveal a new mechanism for regulation of Wnt signaling by Presenilin deficiency.
INTRODUCTION

Canonical Wnt signaling is essential for embryonic development, stem cell and tissue homeostasis, and regeneration in the adult (MacDonald et al., 2009; Angers and Moon, 2009). Aberrant Wnt signaling has been associated with human diseases such as cancer, bone disorders and neurodegeneration (Clevers and Nusse, 2012; Boonen et al., 2008). In the absence of Wnt ligands, the adaptor protein and transcription co-factor β-Catenin is phosphorylated by GSK3 in the destruction complex consisting of the tumor suppressor Adenomatous Polyposis Coli (APC), Axin, Casein Kinase 1 (CK1) and the E3-polyubiquitin ligase βTrCP (Cadigan and Peifer, 2009; Li et al., 2012). Phosphorylations by GSK3 target β-Catenin and other proteins for polyubiquitinylation and degradation in the proteasome (Kim et al., 2009; Taelman et al., 2010; Clevers and Nusse, 2012). Binding of Wnt ligands to their receptors Frizzled (Fz) and LDL-receptor related protein 5/6 (LRP5/6) triggers recruitment of Dishevelled (Dvl), Axin, and GSK3 to the plasma membrane (Bilic et al., 2007; Zeng et al., 2008). GSK3 is first recruited by the binding of Axin to LRP6, and then becomes engaged in the phosphorylation of LRP6, Fz, Dvl, Axin and β-Catenin, which contain multiple GSK3 sites, explaining the requirement of an intact GSK3 catalytic site for its relocalization (Taelman et al, 2010). Wnt receptor complexes, containing Axin and GSK3, are then internalized into the cell by endocytosis (Blitzer and Nusse, 2006; Yamamoto et al., 2006) and subsequently sequestered by incorporation into the intraluminal vesicles (ILVs) of late endosomes that are produced by invagination and scission from the endosomal limiting membrane (Taelman et al., 20120; Dobrowolski and De Robertis, 2012). Sequestration of active GSK3 inside MVEs leads to the sustained stabilization of the half-life of many GSK3 protein substrates (Taelman et al., 2010), principal among which is newly-
synthesized β-Catenin which enters the nucleus to co-activate Wnt target genes.

The integration of cell signaling and endocytosis is critical for signal transduction outcomes (Sorkin and von Zastrow, 2009; Dobrowolski and De Robertis, 2011). While most receptor complexes are negatively regulated by endocytosis (Katzman et al., 2002), Wnt signal transduction requires the function of the endolysosomal pathway (Blitzer and Nusse, 2006). Inhibition of ILV formation in MVEs (also referred to in the literature as multivesicular bodies or MVBs) by interfering with components of the endosomal sorting complex required for transport (ESCRT) (Katzman et al., 2002; Wollert and Hurley, 2010) prevents canonical Wnt signaling (Taelman et al., 2010). Since endolysosomal function is essential for Wnt signaling, we decided to investigate the effect of inhibitors of lysosomal function on Wnt signaling. It was recently reported by Nixon’s group that Presenilin 1 (PS1), an intramembrane protease mutated in early-onset Familial Alzheimer’s disease (FAD), is required for proper autophagosome digestion (Lee et al., 2010). These authors found that the acidification of lysosomes was impaired in PS1-deficient cells, and proposed a model in which Presenilins are required for lysosomal maturation. An extensive literature linking autophagy defects and neurodegeneration exists (Nixon et al., 2008). Furthermore, toxic amyloid precursor protein (APP) peptides accumulate intracellularly specifically in MVBs in early stage Alzheimer’s disease (Takahashi et al., 2002), and certain polymorphisms in the lysosomal protease Cathepsin D increase risk for Alzheimer’s disease (Nixon and Yang, 2011). Taken together, these observations motivated us to investigate whether Presenilin deficiency might affect Wnt signaling through functional changes in the endolysosomal system.
In this study we report that canonical Wnt signaling activity was significantly increased when lysosomal function was inhibited by Chloroquine (CQ), a drug that raises lysosomal pH. Depletion of Presenilin 1 or 2 also resulted in a significant increase of Wnt transcriptional activity in TCF/β-Catenin reporter gene assays. In both cases, lysosomal function was inhibited downstream of ILV formation. Indeed, the ESCRT machinery, which generates ILVs, and the small GTPase Rab7, which is involved in MVE/late-endosome formation (Bucci et al., 2000; Huotari and Helenius, 2011), were both required for Wnt transduction. In electron microscopy analyses, Chloroquine-treated or Presenilin-deficient cells displayed a striking accumulation of late endosomal vacuoles containing intraluminal vesicles. Immunofluorescence microscopy studies indicated that these MVEs were positive for Rab7 and the ILV membrane marker CD63 (Escola et al., 1998). The expanded late endosomes sequestered more GSK3/Wnt-receptor complexes than wild type cells when cells were treated with Wnt. Several GSK3 phosphorylation protein substrates became more stabilized by Wnt in PS1-depleted cells, such as β-Catenin, the previously developed GFP-GSK3-protein stability biosensor (Taelman et al., 2010), and the microtubule-associated protein Tau-GFP. The results presented highlight the key role played by the endolysosomal pathway in Wnt signal transduction.
RESULTS

Chloroquine increases Wnt signaling via MVEs

To investigate the role of the endolysosomal system in Wnt signaling, we used Chloroquine (CQ), an anti-malarial drug. CQ is a weak base that accumulates in lysosomes, causing their alkalinization and the inhibition of the activity of acidic hydrolases (Wibo and Poole, 1974). Sensitive transcriptional reporters of Wnt activity exist, in which multiple T-cell factor (TCF) binding sites (called SuperTopFlash and BAR reporters) activate transcription when \( \beta \)-Catenin accumulates in the nucleus (Veeman et al., 2003; Biechele and Moon 2008). As shown in Figure 3-1A and 3-1B, Wnt signaling was stimulated up to 7-fold by CQ treatment in HEK293T cells (brackets in left). Increases in Wnt signaling were also observed using the lysosomal protease inhibitors Leupeptin or E64 (increases of 2.6 and 2.1 fold, respectively, Supplementary Figure 3-S1A). Therefore, interference with lysosomal maturation and function potentiates Wnt signaling.

To test whether the amplification of Wnt signaling by CQ required formation of intraluminal vesicles in MVEs, we used siRNA knockdown of hepatocyte growth factor regulated Tyrosine kinase substrate (HRS), also known as Vacuolar protein sorting 27 (Vps27), which is required for early stages of ILV formation (Katzmann et al., 2002; Taelman et al., 2010). HRS/Vps27 was required for the stimulation of Wnt signaling by CQ (Figure 3-1A, right bracket). Vps4, another ESCRT component required for ILV formation was also required, as was Rab7, a protein required for late endosomal maturation (Figure 3-1B, right bracket, and data not shown).

To determine whether CQ causes the accumulation of MVEs, we examined 3T3 cells
Fig. 3-1. Lysosomal Inhibition by Chloroquine Increases Wnt Signaling and Expands the MVE Compartment.

(A) CQ (50 μM, Sigma) significantly increases the Wnt responsiveness of HEK293T cells in a Wnt reporter (SuperTopFlash) assay (left bracket, 11.2 ± 2.1 fold increase relative Luciferase Renilla units, RLU, in Wnt treated control siRNA cells vs. 69 ± 8.8 in Wnt treated CQ cells). HRS ESCRT protein is required for this stimulation (left bracket).

(B) Chloroquine stimulation of Wnt activity (BAR Wnt reporter) can be blocked by dominant-negative Rab7 co-transfection (cells treated with Wnt and CQ had a 243 ± 39 fold induction and 55 ± 8.9 RLU after co-expression of Rab7DN).

(C-E) Treatment of 3T3 fibroblasts with CQ for 12 h leads to the formation of numerous endosomal and autolysosomal vesicles bounded by a single membrane, containing intraluminal vesicles (ILVs) of about 50 nm characteristic of MVEs, and electron dense material representing digested cytoplasmic components taken in by microautophagy.
(F and G) After CQ treatment for 15 min, MVEs frequently showed membrane invaginations containing ribosomes and aggregated cytoplasmic components (arrows), reflecting the process of microautophagy.

(H and I) Flow cytometry analyses showing an increase of the late endosomal markers Rab7 and CD63 in cells treated with CQ for 8h. All histograms are presented as mean ± SEM (standard error of the mean). See also Figure 3-S1 and Movie 3-S1.
treated overnight (Figure 3-1C to 3-1E) or L-cells treated with CQ for 6 h, 1 h or 15 min (Figure 3-1F and 3-1G) by transmission electron microscopy. In both cell lines Chloroquine treatment caused a striking increase in autolysosomes containing accumulations of electron-dense cytoplasmic materials, such as aggregated proteins, as well as small intraluminal vesicles of about 50 nm characteristic of MVEs (Figure 3-1D and 3-1E). Doubled-membraned macroautophagic vacuoles were not seen, even at early time points. However, the MVEs were clearly engaged in microautophagy (Sahu et al., 2011) since already after 15 minutes of CQ treatment many showed large invaginations of the limiting membrane (Figure 3-1G, arrows) enclosing regions of cytoplasm containing fine electron-dense granules corresponding to ribosomes. These invaginations, upon pinching off the limiting membrane, generate the electron dense deposits in late autolysosomes, which are enveloped by single or multilaminar membranes, depending on the stage of the individual autolysosomes (Figure 3-1E).

Chloroquine treatment causes the rapid accumulation of endosomes marked in their outer membrane by Rab7 (Figure 3-S1B to 3-S1C’’’ and Movie 3-S1). These vacuoles correspond to MVEs because they colocalize with the tetraspanin protein CD63, a marker for ILV membranes (Figure 3-S1D to 3-S1E”; Escola et al., 1998). Flow cytometry confirmed that CQ-treated cells had increased levels of Rab7 (by 25.2%) and of CD63 (by 28.5%) antigens in the cell population as a whole (Figure 3-1H and 3-1I).

We conclude that the inhibition of lysosomal function caused by Chloroquine does not prevent the formation of endosomal intraluminal vesicles but instead enhances it. The MVE expansion would enhance the sensitivity of cellular responses to Wnt, which are entirely dependent on the ESCRT machinery.
Dose-dependent Response of Wnt Signaling to Chloroquine and Bafilomycin

We next investigated the effects of inhibiting lysosomal function on Wnt signaling with different doses of CQ or the specific vacuolar ATPase (v-ATPase) inhibitor Bafilomycin A (Figure 3-2A and 3-2B). The v-ATPase enzyme acidifies the entire endosomal pathway as vesicles traffic from the plasma membrane to lysosomes. We found that low concentrations of CQ or Bafilomycin increased Wnt3a responses in LSL cells stably transfected with a TCF reporter, while at higher concentrations (>250 µM for CQ and >50 nM for Bafilomycin) both drugs inhibited Wnt signaling, presumably by alkalinizing also the early endosomal compartment (Figure 3-2A and 3-2B). Since the generation of ILVs within the expanded endosomes can still proceed in the presence of CQ (Figure 3-1E), the accumulation of intraluminal vesicles in MVEs may provide the basis for the increased sensitivity to Wnt signals after CQ treatment. At high CQ concentrations, the more extensive alkalinization of the entire endolysosomal pathway may actually inhibit ILV formation or receptor activation. Our results agree with a previous study by Cruciat et al. (2010) who, using HEK293T cells, found that Bafilomycin inhibited Wnt signaling, since LRP6 phosphorylation requires acidification of early endosomes/signalosomes. We now extend their findings by showing that at low doses v-ATPase inhibition increases Wnt signaling, presumably reflecting a higher requirement for the proton pump in the more acidic late endosomal compartment. These results highlight the link between Wnt signal transduction and membrane trafficking.

Presenilin deficiency Leads to Increased Sensitivity to Wnt Signals

A new function for Presenilin in the maturation of v-ATPase was recently reported (Lee et al., 2010), motivating us to explore the impact of lysosomal malfunction caused by Presenilin
Fig. 3-2. Wnt Sensitivity Increases when the Lysosomal Pathway Is Inhibited by CQ, low-dose Bafilomycin, or Presenilin Depletion.

(A and B) Concentration-dependent effects of Chloroquine and Bafilomycin A (Sigma) on Wnt Luciferase reporter assays in LSL-cells (SuperTopFlash reporter). Concentrations of 100 μM for CQ or 2.5 nM for Bafilomycin A caused maximal increases in Wnt signals (145 ± 23 or 162 ± 34 fold increase in RLUs, respectively). Higher concentrations of either drug lead to weaker increases and eventually inhibition of the signal. In the Bafilomycin experiment, all samples contained a final concentration of 0.1% ethanol, which was used as vehicle.
(C and D) PS1 depletion expands the endosomal fluid-phase compartment in DextranRed endocytosis assays in HeLa cells. (E) Flow cytometry quantification confirms expansion of the endocytic compartment marked by DextranRed after PS1 depletion.

(F) Wnt signaling is increased in PS1 depleted HEK293T cells (BAR Luciferase assays). The highest concentration of Wnt3a used had a difference between PS1 siRNA and control siRNA of 3.3 times (743 ± 89 vs. 225 ± 27 fold induction, see bracket).

(G) The enhancing effect of Presenilin depletion on Wnt signaling (of 4.6 times in this experiment) is ESCRT dependent, for it is abolished when HRS is depleted by siRNA in HEK293T cells (173 ± 24 fold increase vs. 1.1 ± 0.2 RLU, right bracket).

(H) The Wnt-enhancing effect of Presenilin knockdown is also observed in Xenopus animal cap explant experiments. PS1 morpholino (MO) increased the Wnt signal by 2-fold (5.6 ± 0.8 vs. 2.8 ± 0.3 RLU, left bracket) and was inhibited when HRS MO was co-injected into early Xenopus laevis embryos (right bracket).

(I) Depletion of PS1, PS2, or both, led to increases of Wnt signaling in Wnt Luciferase reporter assays (BAR reporter) in HEK293T cells (24 ± 3.2 fold for Wnt in control siRNA cells, compared to 86 ± 9.2 for PS1, 69 ± 7.2 for PS2, and 124 ± 14.6 for both PS1 and PS2 siRNA). All values in histograms are presented as mean ± SEM of three independent experiments. See also Figure 3-S2.
deficiency on Wnt signaling. We first confirmed that knock out or siRNA depletion of PS1 indeed generated less acidified endosomes, as was shown by reduced staining with LysoTracker which marks acidic organelles (Figure 3-S2A to 3-S2E’). Efficient knockdown of PS1 was demonstrated with an immunoblot using a Flag-tagged PS1 construct (Figure 3-S2F). We then extended the observations of Lee et al. (2010) by showing that the fluid-phase endosomal compartment, measured by endocytosis of DextranRed (Tetramethyl-Rhodamine Dextran), was significantly expanded by PS1 knockdown with siRNA (Figure 3-2C and 3-2D). Flow cytometry confirmed the increase in DextranRed accessible endosomes in the PS1-depleted cell population in a quantitative way (Figure 3-2E).

Endosomal expansion was accompanied by an increase in Wnt responsiveness in PS1-depleted HEK293T cells, particularly at higher Wnt3a doses (Figure 3-2F, bracket). The stimulation of Wnt signaling caused by PS1 siRNA-mediated depletion was entirely dependent on intraluminal vesicle formation in MVEs since, as shown in Figure 3-2G, HRS/Vps27 depletion strongly inhibited Wnt signaling in control siRNA or PS1 siRNA treated HEK293T cells. We extended these findings to the *Xenopus* animal cap system (Taelman et al., 2010). We designed a PS1 Morpholino (MO) oligo that reproducibly increased signaling by SuperTopFlash reporter in cells coinjected with *xWnt8* mRNA (Figure 3-2H), and this effect was rescued by human Presenilin 1 coinjection (Figure 3-S2G). This increase in Wnt signaling was blocked by HRS/Vps27 MO (Figure 3-2H, right bracket). We conclude that the ESCRT machinery is required for the effects of PS1 knockdown both in cultured cells and in the in vivo animal cap system.

We also tested whether Presenilin 2 (PS2) affected Wnt signaling using siRNA. Depletion of PS1 or PS2 led to increased responsiveness in Wnt Luciferase assays (Figure 3-2I),
indicating that both proteins have functional overlap, at least in HEK293T and HeLa cells (see also Figure 3-3A to 3-3D’’ below). Although the increase in Wnt signaling by PS1 siRNA was higher than that of PS2 siRNA, both together had additive effects (Figure 3-2I). The effects of PS2 siRNA on Wnt signaling, as well as those of PS1, could be rescued by overexpression of the human PS1 gene (Figure 3-S2H). Interestingly, FAD-associated PS1 mutations (M146V, A246E or L392V) were much less effective at rescuing the Wnt signaling increased by PS1 depletion (Figure 3-S2I). In contrast, PS1 mutations (D257A or D385A) in the aspartates required for proteolytic activity were as effective as wild-type PS1 in rescuing the effect on Wnt signaling (Figure 3-S2I). This is in agreement with previous work showing that the effects on Presenilins on autolysosomes are independent of γ-secretase enzyme activity (Lee et al., 2010; Neely et al. 2011). The fact that FAD point mutations behave differently from protease-deficient PS1 could have relevance for disease.

Taken together, these data suggest that Presenilin depletion increases the capacity of cells to respond to Wnt by promoting the generation of ESCRT-dependent ILVs in the endosomal pathway.

**Late Endosomes Accumulate in Presenilin 1 and 2 Deficient Cells**

PS depletion had a dramatic impact on the endolysosomal system. In electron microscopy studies, depletion of PS1, PS2, or the simultaneous depletion of both gene products, resulted in an increase in number and size of single-membrane-bounded vacuolar structures in HeLa cells (compare Figures 3-3A to 3B-3D). These structures were autolysosomes and multivesicular endosomes, as they contain a variable number of small and uniform intraluminal vesicles (Figure 3-3B’ to 3-3D’) as well as electron dense undigested cytoplasmic material. Double-membrane
Fig. 3-3. PS1 or PS2 Depleted Cells accumulate MVE/Autolysosomal vacuoles.

(A-D) Low-power electron micrographs of HeLa cells transfected with control siRNA, or depleted of PS1, PS2, or PS1 and PS2. Note the accumulation of MVEs and autolysosomes containing undigested electron dense material.

(A’-D’) Higher magnification views of the same cells showing autolysosomes containing electron dense material, multilaminar membranes, and intraluminal vesicles (ILVs).

(A”-D”’) Even higher power views of endosomes and endolysosomal vesicles containing large numbers of intraluminal vesicles (white arrowheads indicate limiting membranes).

(E and F) Flow cytometry showing that PS1 depletion increases fluorescence intensity of the late endosome limiting membrane marker Rab7 (mean increase of 30.9%) or the ILV membrane protein CD63 (mean increase of 35.7%) in comparison to control siRNA. See also Figure 3-S3.
macroautophagic vesicles were not observed. Some of the most striking MVEs are shown in Figure 3-3A’’ to 3-3D’’. Immunofluorescence analyses showed increased staining for the endosomal pathway markers Rab7 and LAMP1, as well as the ILV marker CD63 when PS1 or PS1/2 were depleted with siRNA (Figure 3-S3 and data not shown). Flow cytometry showed increased levels of Rab7 (by 30.9%) and CD63 (by 35.7%) in the entire cell population after PS1 knock down (Figure 3-3E and 3-3F).

We conclude that PS1 and PS2 play a fundamental role in endolysosomal biogenesis and function and, as in the case of Chloroquine, Presenilin deficiency blocks the pathway downstream of intraluminal vesicle formation.

**Presenilin Depletion Leads to Increased phospho-LRP6 and nuclear β-Catenin upon Wnt signaling**

Given the expansion of the MVE compartment in PS1 depleted cells, we next asked whether trafficking of the activated Wnt co-receptor LRP6 was affected upon Wnt ligand binding. Phosphorylation of LRP6 marks the initial activation step in the Wnt signaling cascade (Niehrs and Shen, 2010). HEK293T cells stably expressing LRP6-GFP (Kategaya et al., 2009) were transfected with control or PS1 siRNAs, treated with high concentrations of Wnt3a overnight, and activation of LRP6 detected with a phospho-specific anti p-LRP6 antibody (Figure 3-4A to 3-4F). Phospho-LRP6 immunostaining in vesicle-like cytoplasmic puncta (Bilic et al., 2007) was detected only in cells treated with Wnt (arrows), and was significantly higher in cells lacking PS1 (compare Figure 3-4D to 3-4B). Only a subset of cells displayed strong responses to Wnt, probably due to the cell cycle dependence of Wnt signaling (Davidson et al., 2009; Niehrs and Acebron, 2012). Immunoblot analyses confirmed that PS1 knockdown caused
Fig. 3-4. Presenilin Depletion Leads to Accumulation of phosphorylated-LRP6 receptor and nuclear β-Catenin upon Wnt signaling.

(A-D) The Wnt co-receptor LRP6 is phosphorylated specifically upon Wnt addition, and accumulates to a greater extent in PS1-depleted HEK293T cells stably expressing LRP6-GFP. Arrows indicate accumulation of phospho-LRP6 staining in cytoplasmic endosomes (called LRP6-Signalosomes by Bilic et al., 2007).

(E-E’) Phosphorylation of LRP6 increases after Wnt3a treatment of HEK293T cells while total LRP6-GFP levels remain unaffected.

(F) The ratio of pLRP6/LRP6-GFP (normalized to α-Tubulin) was increased by Wnt3a treatment in control siRNA and PS1-depleted cells. Quantitative evaluation from 3 independent western blot experiments using ImageJ software.
(G-J') Total β-Catenin increased with Wnt3a treatment and accumulated in the plasma membrane, cytoplasm, and inside the nucleus in HeLa cells. In the absence of Wnt3a addition, PS1-depleted cells showed a slight increase of β-Catenin in the cytoplasm, but β-Catenin was excluded from the nucleus. Wnt3a treatment of PS1 depleted cells led to a strong accumulation of β-Catenin inside the nucleus and in cytoplasmic puncta. Nuclear localization is indicated by white arrows and is counterstained with DAPI.

(K and K') Immunoblot analyses showing significantly increased β-Catenin levels in Wnt and PS1 siRNA treated cells (lanes 1, 2 and 4, 1.97 ± 0.12 fold and 2.59 ± 0.16 fold, respectively). Resulting mean values presented were obtained from 5 independent western blot experiments.
a reproducible increase in LRP6 phosphorylation in the cell population as a whole, while total levels of non-phosphorylated LRP6-GFP were not affected (Figure 3-4E and 3-4E’). Accordingly, the ratio of pLRP6/LRP6-GFP was increased by Wnt in PS1-depleted cells (Figure 3-4F).

Accumulation of β-Catenin in the nucleus constitutes one of the hallmarks of the Wnt signaling cascade. Nuclear accumulation of β-Catenin was detectable only after Wnt treatment in HeLa cells (Figure 3-4G to 3-4J’, arrows indicate location of individual nuclei). The increase in both nuclear and cytoplasmic β-Catenin was highest in PS1 depleted cells treated with Wnt (Figure 3-4J). The increase in β-Catenin levels was quantified in immunoblot analyses (Figure 3-4K, 3-4K’). A weak but significant increase in β-Catenin was also observed in PS1 deficient cells even in the absence of the Wnt ligand (1.38 ± 0.08 fold increase over control, Figure 3-4K, lanes 1 and 3). However, this increase of β-Catenin in PS1-depleted cells (see also Soriano et al. 2001 and Kang et al., 2002) did not generate a transcriptional Wnt signal (see Figure 3-2F, 3-2G and 3-2I). This is explained by the observed accumulation in the cytoplasm but not in the nucleus in the absence of Wnt (compare Figure 3-4I to 3-4J).

We conclude that PS1 depletion causes increased levels of activated phospho-LRP6 receptor in cytoplasmic puncta (presumably corresponding to endosomal vesicles of the type shown in Figure 3-3) when cells are exposed to Wnt, and that this correlates with increased nuclear β-Catenin accumulation.

**GSK3 Translocates to Endosomes in Presenilin Deficient Cells upon Wnt signaling**

To investigate whether the increase in Wnt signaling observed in CQ treated or PS1 depleted cells could be explained by the GSK3 sequestration mechanism, we used HeLa cells.
transiently expressing GSK3-RFP. Overnight exposure of these cells to Wnt conditioned medium led to a relocalization of GSK3-RFP into cytoplasmic puncta (compare Figure 3-5A to 3-5B), which could be visualized when cells were washed with the mild detergent Saponin to reveal the endocytic compartment prior to fixation (see Extended Experimental Procedures). This re-localization of GSK3 to cytoplasmic puncta upon Wnt signaling was strongly enhanced when cells were treated with Chloroquine (Figure 3-5C and 3-S4A). These Wnt-induced GSK3 puncta colocalized with the late endosome marker Rab7 (Figure 3-5B” and 3-5C’’).

We next used an activated form of Rab5 (Rab5QL-DsRed), which induces formation of large MVBs (Wegener et al., 2010), to show that GSK3-GFP is translocated to endosomes. HeLa cells were transfected with either control or PS1 siRNAs, treated with Wnt3a conditioned medium overnight, and treated with Saponin before fixation. We observed an accumulation of GSK3-GFP puncta inside and in the periphery of Rab5QL MVBs, specifically when PS1-depleted cells were treated with Wnt (compare Figure 3-5F-F” to 3-5G-G’’; see also 3-S4B). We also found that Wnt treatment led to the translocation of GSK3-GFP to MVBs in control siRNA cells (Figure 3-5D-D” and 3-5E-E”; see also 3-S4B), although to a lesser degree.

These results suggest that depletion of PS1 with siRNA or inhibition of lysosome function with Chloroquine enhanced the translocation of GSK3 to late endosomes/MVBs that is normally triggered by Wnt.

**GSK3 Protein Substrates are Stabilized by Wnt in PS1-depleted cells**

Since GSK3 is more efficiently sequestered in MVEs of PS1-deficient cells, we investigated whether GSK3 phosphorylation substrates would be more stable in these cells. Consistent with the GSK3 sequestration model, we observed that cytosolic GSK3 substrates such
Fig. 3-5. Wnt-Induced Translocation of GSK3 is increased by Chloroquine or Presenilin siRNA Treatment.

(A-C") GSK3-RFP expressing HeLa cells were treated with Saponin to visualize endosomal GSK3 and endogenous Rab7 staining. GSK3 signal without Saponin treatment is shown in insets labeled a’-c’'. Note that virtually no endosomal GSK3 staining was detected in control cells; endosomal GSK3 staining was detected when cells were exposed to Wnt3a overnight, and was strongly increased when cells were treated with Chloroquine and Wnt. Magnification of an area in C'' shows partial co-localization of GSK3-RFP with Rab7 antigen (arrows).
(D-G") GSK3-GFP re-localized to Rab5QL-DsRed MVBs in Wnt-treated cells, and this co-localization was strongest when PS1 is depleted (arrows). Experiments carried out with Saponin-treated HeLa cells.
See also Figure 3-S4.
as β-Catenin (Figure 3-4K) and a GFP-GSK3 protein stability biosensor primed by a MAPK site (Figure 3-6A; Taelman et al., 2010) were more efficiently stabilized in Presenilin knockdown cells upon receiving a Wnt signal (Figure 3-6A). As a control, we used a mutated GFP biosensor construct in which the three GSK3 phosphorylation sites were mutated into alanines. It was found that the GSK3-resistant biosensor became stabilized and its levels not affected by Wnt or PS1 siRNA (Figure 3-6B and 3-6B’, compare to 3-6A and 3-6A’). This indicates that the effects of Wnt and PS1 siRNA are exerted at the level of protein half-life via GSK3 sites.

Using the same experimental design, we found that transfected Tau-GFP (Kwan and Kirschner, 2005) was stabilized by Wnt treatment and that this stabilization was more marked when PS1 was depleted (Figure 3-6D and 6D’). Tau is a microtubule associated protein that plays an important role in Alzheimer’s disease and contains multiple GSK3 phosphorylation sites. When Tau phosphorylation by GSK3 was measured in immunoblots using an anti phospho-Tau (T181) antibody, Wnt signaling decreased phosphorylation in both control and PS1-depleted cells (Figure 3-6C and 3-6C’). These results support the hypothesis that Wnt signaling removes active GSK3 enzyme from the cytosol, decreasing protein phosphorylations that target GSK3 protein substrates for degradation. The increased protein stabilization caused by Wnt in PS1-deficient cells, in particular in the case of Tau, could provide a new link between Wnt signaling, protein stabilization and neurodegeneration.
Fig. 3-6. Presenilin Depletion Causes Increased Stability of Wnt-regulated GSK3 Substrates.

(A and A’) The GSK3-GFP biosensor consists of a Flag-tagged GFP containing MAPK-primed GSK3 phosphorylation sites (Taelman et al., 2010), and provides a measure of cytosolic GSK3 activity. Wnt treatment stabilized a GSK3-biosensor in control conditions (lane 2, 1.8 ± 0.08 fold increase over control siRNA) and was more efficiently stabilized in PS1-depleted cells when treated with Wnt (lane 4, 2.6 ± 0.16 fold increase over control).
(B and B') The control GFP biosensor (mutGSK3 biosensor) lacking GSK3 phosphorylation sites showed a higher stability than the GSK3-GFP biosensor protein, but no significant changes in stability were detected when cells were treated with Wnt or PS1 siRNA. This control shows that the stabilization of protein half-life by PS1 depletion and Wnt is mediated by the GSK3 sites.

(C and C') GSK3-specific Tau phosphorylation was decreased by Wnt treatment in control or PS1-depleted cells (decrease to 0.5 ± 0.03 in control or 0.59 ± 0.04 in PS1 siRNA lysates). Specific GSK3 phosphorylation of the endogenous microtubule associated protein phospho-Tau was tested in immunoblots using the pTau (T181) antibody.

(D and D') Total Tau stability determined in Tau-GFP expressing HeLa cells. Tau-GFP was stabilized in control siRNA transfected cells by overnight treatment with Wnt3a (lane 2, 5.9 ± 0.23 fold increase over control). Accumulation of Tau without Wnt was also detected in PS1-depleted cells (lane 3, 3.2 ± 0.13 fold increase over control). The stability of Tau was further increased when PS1 siRNA cells were treated with Wnt (lane 4, 8.3 ± 0.33 fold increase). For statistical evaluation, signals from 3 or more immunoblot analyses were quantified using ImageJ.
DISCUSSION

Late Endosomes are required for Wnt Signaling

Recently we proposed a model for sustained Wnt signaling through sequestration of GSK3 in multivesicular endosomes (Taelman et al., 2010). In this study we investigated how Wnt signal transduction is affected when the function of the endolysosomal pathway is altered by inhibiting lysosomal function with Chloroquine, low doses of Bafilomycin A, the lysosomal protease inhibitors Leupeptin or E64, or Presenilin 1 depletion. We found that lysosomal inhibition caused an expansion of the late endosomal compartment, leading to a more efficient sequestration of GSK3 and the generation of an enhanced Wnt signal. The Chloroquine effect had an absolute requirement for the ESCRT machinery, which is essential for the formation of intraluminal vesicles in the endosomal compartment. The enhanced Wnt signaling caused by Presenilin depletion required Hrs/Vps27 not only in cell culture experiments but also in vivo in explants from Xenopus PS1 morpholino-injected embryos. Figure 3-7 shows a proposed model of how lysosomal inhibition increases Wnt/GSK3 signaling and protein stabilization.

The anti-malarial drug Chloroquine, which alkalinizes lysosomes but still allows the formation of ILVs, caused a great expansion of the late endosomal compartment. Not all signaling pathways were increased by Chloroquine treatment; in the case of Sonic Hedgehog (Shh) signaling by a Luciferase reporter was inhibited by Chloroquine, while Hrs-siRNA increased signaling instead of inhibiting signaling as in the case of Wnt (data not shown). An increase in the fluid-phase of the endosomal compartment (marked by endocytosed DextranRed) was observed after siRNA-mediated depletion of Presenilin 1. This gene has been linked to Alzheimer's disease (AD) and recently found to be critical for lysosomal maturation (Lee et al.,
Fig. 3-7. Model of how Chloroquine or Presenilin depletion affects Wnt signaling by expanding the late endosomal compartment

Lysosomal inhibition by Chloroquine or depletion of Presenilin leads to an accumulation of late endosomal vesicles which, upon Wnt signaling, cause increased sequestration of GSK3 in multivesicular endosomes. Lower levels of active GSK3 in the cytosol during Wnt signaling result in increased stabilization of the half-life of GSK3 protein substrates. Note that the activated Wnt receptor complex consists of multiple GSK3 substrates – such as LRP6, Frizzled (Fz), Dishevelled (Dvl), Axin, and phospho-β-Catenin – leading to sequestration of active GSK3 enzyme molecules bound to its substrates.
PS1 and PS2 are intramembrane proteases that form the catalytic core of the γ-Secretase complex that cleaves the transmembrane domains of a large number of proteins. Presenilins are found in most cellular membranes, including in the ILVs of MVEs (De Strooper and Annaert, 2010). In the experiments reported here, lysosomal inhibition or PS depletion was shown to increase Wnt signaling in an ESCRT-dependent manner, but did not generate a signal in the absence of Wnt ligand. This effect could be due to an expanded surface of the limiting membrane of MVEs, or perhaps to changes in pH within the endosome that could increase the sensitivity of the endosomal membrane to undergo invagination to generate ILVs.

Recently published work by Li et al. (2012) described the formation of active Wnt receptor complexes containing most of the components of the β-Catenin destruction complex, except for the E3 ligase βTrCP. In agreement with that report, we observed that βTrCP does not translocate into LRP6 endosomes, while β-Catenin phosphorylated by GSK3 is sequestered inside these Wnt-specific endosomes (Taelman et al., 2010 and unpublished results). These findings underscore the deep relationship between endosomal trafficking and the Wnt signaling pathway.

Depletion of Presenilin Increases Wnt Signaling

Presenilins have been proposed to be involved in maturation of lysosomes through N-glycosylation of the V0a1 subunit of the v-ATPase complex needed for endosomal acidification (Lee et al., 2010). This mechanism has most recently been questioned (Zhang et al., 2012), and it has been suggested instead that Presenilins are involved in the regulation of the gene network associated with lysosomal biogenesis (Sardiello et al., 2009) affecting autophagy/lysosomal
proteolysis independently of lysosomal acidification (Neely et al., 2011; Zhang et al., 2012). Lee et al. (2010) had observed a decrease of endosomal acidification in LysoTracker staining of PS1 knockout cells and siRNA knockdowns, and we were able to confirm their observations. We also found significant increases of MVE markers like CD63, Rab7 and endocytosed DextranRed in cells depleted of PS1. The effects on lysosomal maturation were independent of γ-Secretase activity (Lee et al., 2010; Neely et. al., 2011; Figure 3-S2I), excluding an effect on Notch or other γ-Secretase-dependent pathways.

The enhancement of Wnt signaling by Presenilin depletion discovered here was entirely dependent on ESCRT machinery (Figures 3-1A, 3-2G and 3-2H). Thus, formation of intraluminal vesicles of late endosomes is required for the enhanced effects of the Wnt ligand. Our observations differ from a report by Kang et al. (2002) who proposed that Presenilin functioned as an alternative scaffold for β-Catenin degradation. Importantly, they found that loss of PS1 stabilized β-Catenin in a Wnt-independent way, while in our experiments addition of Wnt protein was required to trigger Wnt transcriptional activity in cell lines with low levels of endogenous Wnt signaling (Figures 3-2F, 3-2G and 3-2I). We note, however, that in *Xenopus* explants PS1 MO did slightly increase β-Catenin signaling (Figure 3-2H). In addition, PS1 siRNA caused a small but reproducible increase of cytoplasmic β-Catenin levels in HeLa cells (Figure 3-4I and 3-4K’). We suggest that the reported Wnt-independent accumulation of β-Catenin (Kang et al., 2002) might be explained in part by an endogenous autocrine Wnt signal that became enhanced though MVE expansion in PS1 knockout cells.

**Presenilin Depletion Increases Protein Stabilization by Wnt**

Proteins normally degraded in lysosomes are taken up from the extracellular space by
endocytosis, are membrane proteins, or are ingested from the cytosol via autophagy. Upon Wnt signaling, cells experience an additional effect, namely the endosomal sequestration of cytoplasmic GSK3 which is translocated into MVBs together with activated Wnt receptor complexes (Taelman et al., 2010; Dobrowolski and De Robertis, 2012). Here we provided evidence that three cytosolic GSK3 substrates were stabilized by Wnt signaling in PS1-depleted cells: the microtubule-associated protein Tau, β-Catenin, and a GFP-GSK3 biosensor.

The experiments suggest a new mechanism for Wnt signaling in Presenilin-deficient cells. We propose that upon Wnt signaling activated receptor complexes are internalized in endosomes and sequester GSK3 inside the expanded late endosomes together with the activated Wnt receptor complex, which is composed of multiple protein components phosphorylated by GSK3 (Figure 3-7). Once cytoplasmic levels of active GSK3 are sufficiently decreased, GSK3 protein substrates become less phosphorylated, their phosphodegron domains are not recognized by E3 polyubiquitin ligases and GSK3 target proteins become stabilized.

This new mechanism could play a role in Alzheimer's disease (AD), since Presenilin deficiency increases the stability of multiple GSK3 substrates upon Wnt signaling. Some of these substrates, such as the microtubule-associated protein Tau, play crucial roles in the pathogenesis of AD. An extensive literature exists linking Tau and GSK3 signaling (Hall et al., 2000; de Calignon, 2012). A role for Wnt signaling in prevalent neuropsychiatric diseases including AD, schizophrenia and autism has been proposed (De Ferrari and Moon, 2006), but studies performed to elucidate the function of Wnt in neurodegeneration have often generated contradictory results (summarized by Boonen et al., 2009). It is possible that Wnt signaling may result in different outcomes at different stages of AD progression. At early stages of AD, increased sequestration of GSK3 may stabilize many proteins, as shown here for Tau. However, chronic protein
stabilization in PS1-deficient cells might eventually lead to failure of the endosomal pathway and Wnt signaling as the disease progresses. Once GSK3 ceases to be sequestered, elevated levels of cytosolic Tau might be more readily phosphorylated by GSK3, triggering AD pathology. It is possible that repeated Wnt stimulation through the course of a lifetime aging process, in combination with the accumulation of multivesicular endosomes caused by defective trafficking, could result in increased protein stabilization and the eventual accumulation of the neurotoxic Amyloid β42 (Aβ42) peptide (De Strooper and Annaert, 2010).

At early AD stages Aβ42 has been localized to MVBs (Takahashi et al., 2002) and is released from neurons via exosomes (Rajendran et al., 2006) to form extracellular amyloid deposits (Vella et al., 2008). According to the amyloid hypothesis for the onset of AD (Selkoe, 1991; Hardy and Higgins, 1992; Hardy 2009), extracellular amyloid plaque deposits would lead to altered kinase/phosphatase activities and hyperphosphorylation of Tau. Other recent studies suggest that phosphorylated Tau spreads in a prion-like manner through neuronal synaptic circuits (Liu et al., 2012; de Calignon, 2012; Kfoury et al., 2012). In addition, autophagy and lysosomal proteolysis defects have also been proposed to be involved in the pathogenesis of AD (Nixon et al., 2008; Lee et al., 2010).

The experiments presented here demonstrate that decreased Presenilin expression causes an increase in Wnt signaling. The results also show that PS1 depletion causes a considerable expansion of the endosomal system, which results from the inhibition of lysosomal function downstream of intraluminal vesicle formation. The new connection between Wnt signaling and increased stability of GSK3 phosphorylation protein targets in Presenilin-deficient cells could explain why AD neurodegeneration is most severe in the hippocampus, especially in its dentate gyrus, and cerebral pyramidal neurons (Brundin et al., 2010), for it is in these particular cells that
physiological Wnt signaling in the adult brain is maximal (Maretto et al., 2003). We propose that, during the aging process defects in membrane trafficking may increase protein stabilization in the cytosol after repeated stimulation by Wnt signaling, contributing to the formation and deposition of intracellular protein aggregates. Whether the proposed mechanisms play a role in human pathology remains to be determined. In the meantime, the results presented here strengthen the intimate connection between Wnt signaling and the cell biology of intracellular membrane trafficking.
MATERIALS AND METHODS

Cell Culture and Knock-Down Experiments

All cell lines used (HeLa, 3T3, HEK293T and L-cells) were cultured in DMEM complete medium containing 10% FBS, 1% Glutamine and 1% Pen/Strep. For knock-down experiments in cultured cells, siRNAs targeting human PS1, PS2, and HRS were ON-TARGETplus SMARTpool from Thermo Scientific #L-004998, #L-006018, #L-016835, respectively. The non-targeting control-siRNA pool was from the same company, #D-001810. For RNAi depletion experiments, cells were transfected with siRNAs 24 h prior to transfections with DNA. For the rescue experiments we introduced a full-length human PS1 gene (Open Biosystems, EHS1001-33034) into the pCS2+ expression plasmid and transfected 0.3 µg of this DNA into each well of a 12-well plate containing siRNA pretreated cells. Knock-down experiments in Xenopus laevis were conducted using Presenilin 1 morpholino (sequence: TTCACTGGTGTCATTCATATTAGCT, Gene Tools, LLC) and Hrs morpholino (sequence: TGCCGCTTCTCTTCCCATTGCGAA , Gene Tools, LLC).

Immunostaining, Flow Cytometry, and Western Blots

The following primary antibodies were used for immunostainings and flow cytometry: anti-Rab7 rabbit monoclonal (Cell Signaling # 9367) at 1:350; anti-CD63 mouse monoclonal (BD Biosciences #556019) at 1:250; anti-β-Catenin rabbit (SantaCruz H-102) at 1:4000; anti-phospho-S1490-LRP6 rabbit (Cell Signaling # 2568) at 1:500.

For Western blots, the following primary antibodies were used: anti-β-Catenin (Sigma #C2206) at 1:4000; anti-Flag monoclonal (Sigma #F1804) at 1:1500; anti-GFP (Molecular
Probes, #A6455) at 1:500; anti-phospho-LRP6 (Cell Signaling #2568) at 1:500; anti-phospho-Tau (T181) (Cell Signaling #5383) at 1:500; anti-α-Tubulin monoclonal (Calbiochem #CP06) at 1:1500. Secondary antibodies coupled to Infra-Red Dyes (IRDye 680 and IRDye 800) at 1:3000 (LI-COR) were used and western blots were analyzed using a LI-COR Odyssey system.

**Luciferase Assays, LysoTracker and DextranRed Staining, hPS1 mutant Constructs and Time-Lapse Movies**

For luciferase assays, cells were incubated with control or Wnt3a conditioned medium overnight, washed, lysed and measured using the Dual Luciferase Renilla Reporter Assay kit (#E1960) in the Promega Glomax plate luminometer following manufacturer’s instructions. For a single well of a standard 12 well plate cells were grown up to a 80% confluency and transfected with 0.2 µg of BAR-Luciferase, 0.02 µg of CMV Renilla, and 0.3 µg of constructs such as GSK3 biosensor or Tau-GFP supplemented with carrier PCS2 empty vector to a final DNA amount of 1 µg.

Wnt3a conditioned medium, as well as control conditioned medium, were harvested from stably transfected L-cells according to the ATCC protocol (Willert et al., 2003), except for using a lower 2% FBS concentration, which we found to be more reliable and active. For the experiments in Figure 4, Wnt conditioned medium was supplemented by the addition of 80 ng/ml of purified Wnt3a protein (PeproTech, NJ). Chloroquine diphosphate (Sigma) was dissolved in H₂O as a 100 mM stock solution and added to cell cultures at about 80% confluency. Transfections of DNA constructs were performed using BioT (Bioland); for siRNAs reverse transfections Lipofectamine 2000 (Invitrogen) was placed first into the plates and mixed with trypsinized cells. For visualization of the endocytic compartment, living HeLa cells were
treated with 0.01% (weight/vol) Saponin in PBS, gently shaking for 5 min at room temperature prior to fixation (Bishop and Woodman, 2000). Endosomal staining (Figure S4) was quantified using the ImageJ software and ITCN plugin that measures intensity maxima from images based on individual parameters (http://www.bioimage.ucsb.edu). For LysoTracker staining, living cells were incubated in pre-warmed culture medium containing diluted LysoTracker Red DND-99 reagent (1:1000, Invitrogen #L7528) for 1 to 3 min at 37°C (depending on the cell line), immediately and quickly washed twice with PBS and fixed with 4% PFA for 15 min at room temperature. Other procedures were as described in Taelman et al., 2010.

For DextranRed staining, HeLa cells were grown on clean glass cover slips, transfected with the appropriate siRNAs and incubated with 2 mg/ml dextran Tetramethylrhodamine (D1868, Invitrogen, NY) diluted in DMEM complete medium for 30 min. Subsequently, cells were washed, fixed with 4% Paraformaldehyde in PBS and mounted on glass slides for microscopic evaluation.

Stably transfected LSL-cells containing SuperTopFlash-Luciferase constructs (Blitzer and Nusse, 2006) and HEK-293T cells stably expressing LRP6-GFP (Kategaya et al., 2009) fusion proteins were generously provided by Drs. Roel Nusse and Randall Moon, respectively.

Human Presenilin 1 (PSEN1, transcript variant 1) from an EST was subcloned, together with a C-terminal Flag-tag into the expression vector pCS2 for rescue experiments. The hypomorphic FAD mutations A246E, L392V and M146V (Lee et al. 2010; Boonen et al. 2009), and the aspartate protease-inactive mutants D257A and D385A (Lee et al. 2010) were introduced by site-directed mutagenesis using Quick-Change II XL kit from Stratagene.

For filming in vivo time-lapse movies with Rab7-GFP and Chloroquine, transfected NIH/3T3 cells were transferred to Lab-Tek coverglass chambers (#155380, Nalge Nunc
International, New York). Pictures were taken every 5 minutes using a Zeiss Observer.Z1, equipped with Apotome.2, fully automated-stage and Temp/CO₂ Module S, using a Colibri 488 LED or DIC optics with an EC Plan-Neofluar 40x/0.75 M27 objective. Chloroquine was added just before acquisition started.

**Electron Microscopy**

Confluent cultures of 3T3 cells in 10 cm dishes treated with 50 µM Chloroquine for the indicated times or of HeLa-cells depleted of Presenilin 1 and/or 2 with siRNA were fixed by replacing the medium with fixative solution (2% glutaraldehyde in 0.1 M sodium Cacodylate buffer pH 7.4). After incubation in cold fixative for one hour, the cells were scraped from the dishes using a Teflon scraper, collected in Eppendorf tubes and recovered by centrifugation for 10 min 10,000 rpm. Cell pellets remained under the fixative for additional 3 hours at RT and were carefully washed three times for 10 min each with 0.1 M Cacodylate buffer. The pellets were post fixed with 2% Osmium tetroxide and processed for embedding in epoxy resin LX 112. Thin sections were stained with Uranyl acetate and Lead citrate and examined by TEM in a JEOL EX 1200 electron microscope.

**Statistics**

Results of three or more independent experiments are given as the mean ± standard error of the mean (SEM). Statistical analyses were performed with Excel (Microsoft Co) applying the two-tailed t test, as appropriate. Significant differences of means are indicated as *≤0.05, **≤0.01, ***≤0.005.
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Fig. 3-S1. Chloroquine Treatment Expands the Rab7 and CD63 Positive Endosomal Compartment, Related to Figure 3-1

(A) Wnt signaling is increased in cells treated overnight with the lysosomal proteases inhibitors Leupeptin (122 ± 12 fold induction) or E64 (107 ± 10) (both obtained from Sigma and used at a concentration of 200 µM or 6 µM in DMSO, respectively) as measured in LSL cells stably transfected with the Wnt reporter SuperTopFlash. (B-B'') Time-lapse microscopy of HeLa cells showing an accumulation of the late endosomal marker Rab7-GFP visible after about 30 min of Chloroquine (CQ) treatment. (C-C'') No changes in Rab7 were detected in control cells. Insets show the signal detected in the Rab7-GFP channel, without Nomarski optics. (D-D'') Endogenous CD63 protein marks the membranes of intraluminal vesicles (ILVs) in multivesicular bodies and to lesser extent lysosomes (Escola et al., 1998). Note that CD63 puncta
are increased in CQ treated cells. Small endosome-like structures could be detected simply by DIC (differential interference contrast) optics when cells were treated with CQ. (E-E”’) Control HeLa cells showing low endogenous levels of CDE63 antigen.
Fig. 3-S2. Decreased Acidification in Presenilin-deficient Cells, and Rescue of PS Knock-Down by Overexpression of wt or Protease-inactive hPS1 but only partially by hypomorphic FAD PS1 Mutations, Related to Figure 3-2
(A-B’) LysoTracker staining showing a marked decrease of acidified endolysosomal vesicles in blastocyst derived PS1 knock-out cells (PS1KO). This result confirms the observations of Lee et al., 2010. (C-E’) LysoTracker staining was weaker in Presenilin-depleted cells indicating impaired acidification, and was virtually absent in cells treated with Chloroquine. (F) Flag-PS1 protein is effectively depleted by PS1 siRNA, but not by control siRNA in transfected HeLa cells; α-tubulin serves as loading control. (G) The increase in Wnt signaling caused by PS1 MO in Xenopus animal cap explants was rescued by coinjection of 20 pg of human PS1 DNA.
(bracket). Relative Luciferase units using the SuperTopFlash reporter, n=2. (H) The increase in Wnt signaling caused by siRNA depletion of PS1, PS2, or both, could be rescued by overexpression of human PS1 (Wnt Luciferase BAR reporter assays in HEK-293T cells). Note that the effect of PS2 or PS1, PS2 depletion on Wnt signaling could be reversed by overexpression of PS1. This experiment shows that the effects of the Presenilin siRNAs used in this study are specific. (I) Overexpression of Presenilin FAD mutations associated with early-onset familial Alzheimer’s disease (M146V, A246E, L392V) did not efficiently rescue the effect of Presenilin depletion in cultured cells, while the aspartate mutations D257A and D385A reversed the effect of PS1 siRNA. All cells were transfected with PS1 siRNA and treated either with control or Wnt conditioned medium and analyzed using SuperTOPFlash-transfected HeLa cells. These results indicate that FAD mutations were less effective than wt or protease-inactive PS1 mutants. The protease activity of PS1 is not required for its effect on Wnt signaling.
**Fig. 3-S3. Presenilin Depletion Enlarges the Multivesicular Body Compartment, Related to Fig. 3-3**

(A-A'''') CD63 and Rab7 puncta co-localize in control untreated HeLa cells. (B-B'''') PS1 depletion with siRNA causes enlarged Rab7 positive vacuoles, some of which contain the ILV marker CD63 in their lumen. Note that the multivesicular endosomes enlarged by PS1 depletion are readily visualized by differential interference contrast (DIC) optics. (C-C'''') Greatly enlarged vacuoles by double knockdown of PS1 and PS2. Note in the inset Rab7 staining surrounding the enlarged endosomes and CD63 localizing to the lumen of these vesicles.
Fig. 3-S4. Quantification of Wnt-dependent relocalization of GSK3 after lysosomal inhibition by Chloroquine or PS1-siRNA in Saponin-permeabilized HeLa cells, Related to Figure 3-5

(A) Endosomal GSK3 staining was increased (6.1±1.6 fold) following Wnt3a treatment. In the presence of Chloroquine, the GSK3 relocalization caused by Wnt treatment was increased further (19.5±5.6 relative to control). (B) Endosomal GSK3 increased (4.1±0.7 fold) by Wnt treatment, while in PS1-depleted cells Wnt further enhanced GSK3 translocation (11.6±1.9 fold). GSK3 signals were quantified as described in Extended Experimental Procedures.
Chapter 4

MITF drives endolysosomal biogenesis and potentiates Wnt signaling in melanoma cells

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ABSTRACT

Canonical Wnt signaling plays an important role in development and disease, regulating transcription of target genes and stabilizing many proteins phosphorylated by Glycogen Synthase Kinase 3 (GSK3). We observed that the MiT family of transcription factors, which includes the melanoma oncogene MITF and the lysosomal master regulator TFEB, had the highest phylogenetic conservation of three consecutive putative GSK3 phosphorylation sites in animal proteomes. This prompted us to examine the relationship between MITF, endolysosomal biogenesis and Wnt signaling. Here we report that MITF expression levels correlated with the expression of a large subset of lysosomal genes in melanoma cell lines. MITF expression in the Tetracycline-inducible C32 melanoma model caused a marked increase in vesicular structures, and increased expression of late endosomal proteins such as Rab7, LAMP1, and CD63. These late endosomes were not functional lysosomes as they were less active in proteolysis, yet were able to concentrate Axin1, phospho-LRP6, phospho-β-Catenin, and GSK3 in the presence of Wnt ligands. This relocalization significantly enhanced Wnt signaling by increasing the number of multivesicular bodies (MVBs) into which the Wnt signalosome/destruction complex becomes localized upon Wnt signaling. We also show that the MITF protein was stabilized by Wnt signaling, through the novel C-terminal GSK3 phosphorylations identified here. MITF stabilization caused an increase in MVB biosynthesis, which in turn increased Wnt signaling, generating a positive feed-back loop that may function during the proliferative stages of melanoma. The results underscore the importance of misregulated endolysosomal biogenesis in Wnt signaling and cancer.
SIGNIFICANCE

MITF, a master regulator of melanocytes and a major melanoma oncogene amplified in 30-40% of melanomas, determines proliferative or invasive phenotypes. Previously unrecognized as a driver of lysosomal biogenesis, we found that MITF expression correlates with many lysosomal genes and generates late endosomes that are not functional in proteolysis. This accumulation of incomplete organelles expands the late endosomal compartment, enhancing Wnt signaling by entrapping the Wnt machinery in MVBs. Wnt signaling can stabilize many proteins besides β-Catenin. Our study identifies MITF as an oncogenic protein stabilized by Wnt, and describes three novel GSK3-regulated phosphorylation sites in this oncogene. This study deepens our knowledge on proliferative stages of melanoma: MITF, MVBs and Wnt may form a feedback loop that drives proliferation.
INTRODUCTION

Wnt signaling is required for tissue differentiation, growth and homeostasis (MacDonald et al., 2009; Angers and Moon, 2009). Misregulation of Wnt signals can result in abnormal development and disease, most notably cancer (Clevers and Nusse, 2012). The canonical Wnt pathway influences transcription through the stabilization of β-Catenin, a transcriptional activator. In the absence of Wnt ligands, β-Catenin is rapidly turned over by a destruction complex composed of Adenomatous Polyposis Coli (APC), Axin1, Casein Kinase 1 alpha (CK1α), and GSK3 (Cadigan and Peifer, 2009). During Wnt signaling, the destruction complex is inhibited, allowing newly synthesized β-Catenin to accumulate in the nucleus and regulate transcription of Wnt target genes (Peifer et al., 1994). However, Wnt signaling stabilizes many other cellular proteins in addition to β-Catenin (Taelman et al., 2010; Acebron et al., 2014). Wnt achieves this stabilization of proteins by inhibiting GSK3, a kinase that generates phosphodegrons in β-Catenin and many other proteins. These phosphodegrons are then recognized by E3 ubiquitin ligases and polyubiquitinated, targeting proteins for proteosomal degradation (Acebron et al., 2014). Upon Wnt binding, the Wnt receptor Low density lipoprotein receptor-related protein 6 (LRP6) recruits the β-Catenin destruction complex including Axin1, GSK3, and phospho-β-Catenin which are endocytosed as “Wnt/LRP6 signalosomes” (Bilic et al., 2007) and translocated from the cytosol into multivesicular bodies (MVBs) (Taelman et al., 2010; Vinyoles et al., 2014). Wnt signal transduction requires an intact Endosomal Sorting Complexes Required for Transport (ESCRT) machinery (Taelman et al., 2014), which is required for the formation of the intraluminal vesicles of late endosomes (Wollert and Hurley,
In this way, Wnt signaling causes GSK3 and Axin1 to become sequestered from their potential cytosolic substrates inside membrane-bounded organelles (Taelman et al., 2010; Vinyoles et al., 2014). This mechanism, which results in the stabilization of many proteins by decreasing the polyubiquitination triggered by the generation of GSK3-induced phosphodegrons, has been recently designated “Wnt-dependent STabilization Of Proteins” or Wnt/STOP (Acebron et al., 2014).

We became interested in MITF when a bioinformatic screen of the human proteome for putative GSK3 targets containing 3 or more consecutive phosphorylation sites followed by a priming site revealed that a remarkable 20% of the human proteome contained such sites (Taelman et al., 2010). Among these, the highest score for phylogenetically conserved putative GSK3 phosphorylation motifs (http://www.hhmi.ucla.edu/derobertis/EDR_MS/GSK3%20Proteome/Table_1-full_table.xls) belonged to a group of basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factors called the MiT family (Hemesath et al., 1994). The four members of this family (MITF, TFEB, TFE3 and TFEC) possess three putative GSK3 sites followed by a priming site close to the carboxy terminus. MiT genes behave as oncogenes (Davis and Fisher, 2007; Haq and Fisher, 2011). In the case of MITF and TFEB, the priming sites were shown to be phosphorylated by RSK1/p90 and PKCβ, respectively (Wu et al., 2000; Ferron et al., 2013), but it was not known whether the new GSK3 sites were phosphorylated.

MITF stands for Microphthalmia Associated Transcription Factor, because mutations in the mitf gene give rise to smaller eyes (Packer, 1967), due to defects in the development and function of the retinal pigment epithelium (RPE) (Hodgkinson et al., 1993; Steingrímsson et al., 2004). MITF is also a melanocyte master regulator gene and a melanoma oncogene.
A point mutation in MITF that inhibits sumoylation and leads to increased activity predisposes to familial melanoma, indicating that MITF is indeed a melanoma oncogene (Yokoyama et al., 2011). In addition, the MITF gene is amplified in 30-40% of melanomas (Garraway et al., 2005). MITF is expressed in many tissues and is subject to alternative splicing and differential promoter usage, giving rise to multiple tissue-specific isoforms. MITF-M, also known as variant 4, is an isoform with an N-terminal truncation that is specifically expressed in melanocytes and melanomas (Fuse et al., 1996; Steingrímsson et al., 2004).

Another important member of the MiT family is TFEB, which is the master regulator of lysosome biogenesis (Sardiello et al., 2009). TFEB binds to a specific DNA sequence known as the Coordinated Lysosomal Expression and Regulation element (CLEAR element) in the promoter region of many lysosomal genes (Sardiello et al., 2009). Regulation of lysosome biogenesis is controlled by the mTOR pathway through phosphorylations of TFEB that retain this transcription factor in the cytoplasm (Roczniak-Ferguson et al., 2012; Settembre et al., 2012), coupling lysosomal biogenesis to nutritional sensors. TFE3 has also been shown to act in a similar manner, promoting autophagy and lysosomal biogenesis (Martina et al., 2014).

Although MITF has not, to our knowledge, been recognized as participating in lysosomal biogenesis (Sardiello et al., 2009; Martina et al., 2014), it is strongly expressed in cell types with high levels of lysosome-related organelles such as melanocytes, osteoclasts, mast cells, and RPE (Steingrímsson et al., 2004).

In the present study, we report that MITF mRNA expression levels significantly correlated with the expression of lysosomal gene transcripts in a large panel of melanoma cell lines. MITF upregulated many, but not all, lysosomal genes in an inducible MITF melanoma
model, and activated transcription of a CLEAR element synthetic promoter. MITF not only induced lysosomal gene transcripts, but also protein markers of late endosomal trafficking and acidic organelles. However, this marked increase in late endosomes failed to increase overall lysosomal degradation of endocytosed bovine serum albumin (BSA). Previous work from our laboratory had shown that expansion of late endolysosomal structures (via Chloroquine treatment or Presenilin knockdown) enhanced Wnt signaling by increasing relocalization of GSK3 into MVBs (Dobrowolski et al., 2012). Induction of MITF expression in a melanoma model, in addition to increasing late endosomal vesicles, also increased Wnt signaling in an ESCRT-dependent manner. In the presence of Wnt, the MITF-induced vesicular structures contained Axin1, GSK3, phospho-β-Catenin, and phospho-LRP6. Wnt prolonged the half-life of MITF protein, and enhanced MITF activity in cultured cells and in Xenopus embryo explants. A custom-made anti-phospho antibody confirmed that the novel C-terminal sites on MITF were indeed phosphorylated by GSK3. The results suggest a positive regulatory loop by which MITF expands MVBs/late endosomes, resulting in increased Wnt signaling which in turn stabilizes MITF by decreasing its GSK3 phosphorylations.
RESULTS

**MITF has Three Consecutive Putative GSK3 Sites**

The best characterized members of the MiT family of bHLH-Zip transcription factors are MITF, the melanocyte master regulator and melanoma oncogene (Steingrímsson et al., 2004) and TFEB, the master coordinator of lysosomes and cellular clearance pathways (Sardiello et al., 2009; Settembre and Ballabio, 2011). Although posttranslational modifications had been extensively studied in this family, a bioinformatics screen discovered three previously unrecognized putative GSK3 phosphorylation sites at their C-terminus (Fig. 4-1A) (Taelman et al., 2010). The high degree of conservation of these serines during evolution (Fig. 4-1B) suggested that these sites might be important for protein function. GSK3 prefers pre-phosphorylated “primed” substrates, phosphorylating Ser or Thr at position -4 (S/TXXXX/T[PO3]) (Cohen and Frame, 2001). Importantly, in the case of MITF and TFEB the Serine residues that could serve as the priming phosphate for GSK3 had been previously demonstrated to be phosphorylated *in vivo* (Wu et al., 2000; Ferron et al., 2013). This prompted us to investigate whether MITF was regulated by Wnt, and whether MITF, like TFEB, also had a role in endolysosomal biogenesis.

**MITF Expression Levels Correlate with many Lysosomal Genes in Melanomas**

Although MITF and TFEB share extensive sequence homology, MITF had not been previously linked to lysosomal biogenesis (Sardiello et al., 2009). We investigated whether melanomas in which the *MITF* gene was amplified had increased transcription of lysosomal
Fig. 4-1. *MITF* mRNA correlates with lysosomal gene expression in melanoma cell lines.

(A) MITF and the lysosomal master gene regulator TFEB have three putative C-terminal GSK3 phosphorylation sites, with a previously validated priming site.

(B) The C-terminal GSK3 sites on MITF have been highly conserved throughout evolution, including in the oriental fruit fly *B. dorsalis*.

(C) Heat map obtained from a RNA microarray panel of 51 melanoma cell lines, which cluster...
into 2 distinct groups, one with high MITF and the other with low MITF expression, when queried for a panel of 89 lysosomal genes. The group with high MITF expression (which includes all cell lines with MITF genomic amplifications) upregulates many, but not all, lysosomal genes (dashed line).

(D and E) Gene Set Enrichment Analysis (GSEA) of an expression data set consisting of a panel of 83 additional, different, melanoma lines confirms that MITF, but not TFEB, significantly correlates with the lysosomal gene set in melanoma. Microarray data for melanoma cell lines was obtained from Hoek et al., 2008. Genes were ranked by their correlation (cor) with MITF or TFEB (red to green = high to low correlation). The positions of lysosomal genes (Sardiello et al., 2009) among over 12,000 genes per cell line are marked as vertical lines (GS). Enrichment of the lysosomal gene set at the top of the ranked lists was assessed with a permutation based Kolmogorov-Smirnoff (KS) non-parametric rank test. A significant correlation for MITF, but not for TFEB, was found.

(F) Transfection of MITF activated a CLEAR element-Firefly luciferase reporter (Sardiello et al., 2009), in transient transfections of HEK 293T cells. Renilla luciferase driven by the CMV promoter was used for normalization purposes.
genes. We first analyzed a panel of RNA microarray data for 51 melanoma cell lines generated at UCLA. Cell lines were allowed to sort out according to their levels of expression of a set of 89 lysosomal genes (Sardiello et al., 2009) using the Rosetta Resolver gene expression data analysis system. As shown in Fig. 4-1C, the melanoma lines clustered into two distinct groups: one with high *MITF* expression and the other with low *MITF* expression. The group with high *MITF* expression (indicated in red in the vertical axis of Fig. 4-1C) was composed mainly of melanoma cell lines harboring genomic *MITF* amplifications. Many, but not all, lysosomal genes were upregulated in the lines expressing high levels of *MITF*. When the same samples were specifically queried for the expression of a subset of 63 lysosomal genes that contain a CLEAR response element within their promoter region (Sardiello et al., 2009), the melanoma lines also strongly clustered into two groups, one with high *MITF* mRNA expression and DNA amplification, and another with low *MITF* (Fig. S1).

These results suggested a positive correlation between *MITF* levels and lysosomal gene expression. To confirm this finding, we performed Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) on the lysosomal gene set using publicly available microarray expression profile data sets for melanoma (Philadelphia, Zurich and Mannheim data sets) (Hoek et al., 2008), encompassing a different group of 83 melanoma lines (i.e., in addition to the 51 lines described above). GSEA revealed that *MITF* transcripts significantly correlated with the expression of the lysosomal gene set in melanomas (p<0.001) (Fig. 4-1D). Surprisingly, *TFEB*, the master regulator of lysosomes, did not correlate with the lysosomal gene set (Fig. 4-1E). This indicated that the upregulation of lysosomal genes observed in melanomas with high levels of *MITF* is not mediated by a secondary increase in *TFEB* expression levels.

To test whether MITF could directly activate the CLEAR element, we co-transfected
HEK 293T cells with a CLEAR element luciferase reporter (Sardiello et al., 2009) and MITF. Co-transfection with TFEB was used as a positive control. We found that MITF, like TFEB, could significantly activate the synthetic CLEAR element reporter (Fig. 4-1F). We conclude from these experiments that MITF expression can drive the transcription of many, but not all, CLEAR element lysosomal genes in melanomas.

**MITF Expands the Late Endolysosomal Compartment**

To further test the effect of MITF on lysosomal biogenesis, we took advantage of the C32 melanoma cell line model (Yokoyama et al., 2011), which has undetectable levels of endogenous MITF, but contains a Tetracycline-inducible MITF-M (Fig. 4-2A). After Tetracycline (Tet) treatment MITF was strongly expressed, as detected by an anti-MITF monoclonal antibody (Fig. 4-2B). In response to MITF induction, C32 cells underwent a phenotypic switch displaying a striking increase in large vesicular structures visible by DIC (Differential Interference Contrast) light microscopy (Fig. 4-2C-D’, arrows). MITF induction also increased the lysosomal membrane marker Lysosomal Associated Membrane Protein 1 (LAMP1) as detected by immunofluorescence (Fig. 4- 2E and F), and this increase could be quantified by flow cytometry (Fig. 4-2G). In addition, CD63, a tetraspanin protein that marks the intraluminal vesicles of MVBs, was also enriched in MITF induced-vesicles (Fig 4-2 H-J). These two endolysosomal markers are themselves lysosomal genes whose promoters contain CLEAR elements, and were found to be highly correlated with MITF in the heat map of Fig. 4-1C.
Fig. 4-2. Tet-inducible MITF expression increases late endolysosomal vesicles in the C32 melanoma cell line.

(A) Schematic diagram of the C32 Tet-inducible MITF melanoma cell line (Yokoyama et al., 2011).
(B) Strong induction of MITF after Tet treatment was detected in C32 cells by western blot with anti-MITF antibody.
(C–D’) Increase in the number of vesicular structures upon MITF induction observed by DIC
light microscopy. Note vesicular structures seen at high power (arrows).

(E and F) MITF induction increases immunostaining of the late endosomal marker LAMP1.

(G) Quantification by flow cytometry of the increase in LAMP1 levels upon MITF induction.

(H and I) MITF induction increases immunostaining of the MVB marker CD63.

(J) Quantification by flow cytometry of the increase in CD63 upon MITF induction.

(K) MITF induction increased the transcripts of many lysosomal genes containing CLEAR elements in C32 melanoma cells, as validated by RT-qPCR. MITF induction in C32 melanoma cells upregulated transcripts for the CLEAR element lysosomal genes $\alpha$-N-acetylglucosaminidase (*NAGLU*), chloride channel voltage sensitive 7 (*CLCN7*), prosaposin (*PSAP*), cathepsin D (*CTSD*), cathepsin A (*CTSA*), sialidase 1 (*NEU1*), $\alpha$-galactosidase (*GLA*), mucolipin 1 (*MCOLN1*), $\beta$-glucocerebrosidase (*GBA*), and serine carboxypeptidase 1 (*SCPEP1*). Error bars indicate the standard error of the mean from three independent experiments.
Using RT-qPCR, we validated that expression of multiple lysosomal mRNAs increased by MITF induction in C32 cells. These included α-N-acetylglucosaminidase (NAGLU), chloride channel voltage sensitive 7 (CLCN7), prosaposin (PSAP), cathepsin D (CTSD), cathepsin A (CTSA), sialidase 1 (NEU1), α-galactosidase (GLA), mucolipin 1 (MCOLN1), β-glucocerebrosidase (GBA) and serine carboxypeptidase 1 (SCPEP1) (Fig. 4-2K). As a negative control, hypoxanthine phosphoribosyltransferase 1 (HPRT1) was shown not to change upon MITF induction in C32 melanoma cells. The ability of MITF to upregulate lysosomal genes was not confined to melanoma cells, as HEK 293T cells transiently transfected with MITF also had increased transcripts for CTSA, MCOLN1, PSAP, GNS, SCPEP1, NEU1, and GLA (Fig. 4-S2). TFEB, which contains a CLEAR element in its own promoter as part of a positive feed-back mechanism (Settembre et al., 2013), was also induced by MITF (Fig. 4-S2), but was not correlated with lysosomal gene expression in the GSEA analysis of melanoma lines (Fig. 4-1E). F-box protein 11 (FBX011), used in this case as a negative control, was not induced by MITF in HEK 293T cells (Fig. 4-S2).

In addition, the vesicles induced by MITF were strongly enriched in the late endosomal marker Rab7 (Fig. 4-S3). Although this small GTPase is not itself considered a CLEAR network lysosomal gene, Rab7 has been recently shown to play a key role in the regulation of melanoma proliferation by exploiting a lineage-specific endolysosomal pathway wiring (Alonso-Curbelo et al., 2014).

TFEB elicits a coordinated response to nutritional and homeostatic cellular demands by regulating the synthesis of lysosomes (Palmieri et al., 2011). By using LysoTracker dye, a specific marker of acidic organelle compartments, we found that MITF induction in C32 cells increased the number of acidic organelles detected by fluorescence microscopy and flow
We next tested whether these MITF-induced vesicles were functional lysosomes. To this end, we used the dequenched Bovine Serum Albumin (BSA-DQ) reagent (Fig. 4-3D). BSA-DQ is normally self-quenched, as a result of heavy labelling by BODIPY dyes. When added to the culture medium, BSA-DQ is incorporated into the liquid phase cellular endosomal compartment by non-receptor-mediated endocytosis. Upon fusion with endolysosomes, BSA-DQ is digested into smaller fragments, relieving the self-quenching and causing a fluorescent signal (Fig 4-3D). Although MITF induction greatly expanded the endolysosomal system, increased acidic organelles, and upregulated the transcription of many lysosomal markers, we were surprised to find that lysosomal activity was not increased, but instead moderately decreased (Fig. 4-3E). This is likely due to the inability of overexpressed MITF to induce the complete repertoire of lysosomal genes, as reflected in the heat map in Fig 4-1C. We conclude that MITF expression in the C32 melanoma model expands late endosome/MVB vesicles but not the number of functional lysosomes.

MITF Enhances Wnt Signaling

The increase in late endosome/MVB structures was reminiscent of the effects of Chloroquine (CQ) or Presenilin depletion. We had shown in a previous study that in these conditions Wnt signaling was enhanced through the expansion of the MVB compartment and subsequent increased sequestration of GSK3 (Dobrowolski et al., 2012). This prompted us to test whether the responsiveness to the Wnt pathway, which is critical in melanoma (O’Connell and Weeraratna, 2009), was affected by MITF expression.
Fig. 4-3. MITF expands acidic organelle compartments but does not increase lysosomal activity.

(A and B) Acidic organelles visualized by treatment of living cells with LysoTracker dye. 

(C) Quantification by flow cytometry of the increase in acidic organelles observed upon MITF induction.

(D) Schematic representation of the BSA-DQ reagent used for detecting lysosomal proteolytic activity. BSA-DQ added to the culture medium is endocytosed, but only fluoresces when cleaved by proteases inside lysosomes.

(E) MITF induction decreases lysosomal activity, as quantified by flow cytometry.
The *Xenopus* system provides an efficient way of analyzing Wnt signaling. We found that *MITF* mRNA microinjections significantly potentiated Wnt8 signaling in animal cap ectodermal explants (Fig. 4-4A), using a SuperTopFlash-Luciferase Wnt reporter as the assay (see methods). In accordance to the GSK3 sequestration model (Taelman et al., 2010; Vinyoles et al., 2014), this *MITF*-driven enhancement of Wnt activity required the ESCRT machinery components Hepatocyte growth factor-Regulated tyrosine kinase Substrate (HRS, also known as Vps27) and vacuolar protein sorting protein 4 (Vps4) (Fig. 4-4A). In addition, *MITF* mRNA microinjection expanded the expression domain of *chordin*, a downstream target of Wnt in the developing *Xenopus* gastrula, when coinjected with suboptimal amounts of Wnt8 mRNA (Fig 4-4B-E, arrows). These results showed that *MITF* expression enhanced the Wnt signaling response in the *Xenopus* system, and that this required MVB formation.

To study the effect of MITF on Wnt signaling specifically in a melanoma background, we generated permanent lines of MITF-inducible C32 cells transduced with the Wnt/β-Catenin-activated reporter (BAR) firefly Luciferase reporter together with a Renilla Luciferase driven by an EF1α promoter for normalization purposes (see methods). Using this cell line, we found that MITF induction with Tet significantly enhanced the response to the addition of Wnt3a protein (Fig. 4-4F, brackets). HRS/Vps27 was required for Wnt signaling in this system as well, since HRS siRNA inhibited Wnt signaling (Fig. 4-4G). LiCl is a direct inhibitor of GSK3 that stabilizes β-Catenin and this effect is expected to be independent of endolysosomal organelles. In agreement with this prediction, MITF had no significant effect on LiCl-induced Wnt reporter activity (Fig. 4-4H). As mentioned above, CQ treatment enhances Wnt signaling by accumulating late endosome/MVB structures (Dobrowolski et al., 2012). In C32 melanoma cells, CQ also enhanced Wnt3a signaling, and the extent of its effect was comparable to that of MITF.
Fig. 4-4. MITF enhances Wnt signaling in Xenopus and in melanoma cells in an ESCRT-dependent manner, and causes increased MVB localization of the destruction complex component Axin1.

(A) MITF enhances Wnt signaling in *Xenopus* ectodermal explants. This enhancement is ESCRT-dependent, as it is blocked by HRS/Vps27 morpholino, or a dominant-negative form of the Vps4 ATPase (HRS MO and VPS4E) (**p < 0.001).

(B-E) MITF cooperates with a low dose of Wnt8 mRNA, expanding the Spemann organizer (arrows), the region that expresses chordin mRNA in *Xenopus* whole-mount in-situ hybridization.

(F) MITF induction increases Wnt signaling in the C32 MITF-inducible melanoma cell line. The Wnt BAR Firefly luciferase reporter and EF1α-driven Renilla luciferase by were permanently introduced with lentivectors into the C32 cell line.

(G) HRS/Vps27 knockdown by HRS siRNA decreased Wnt signaling in Tet-induced C32 cells (**p < 0.01).

(H) MITF induction did not affect Lithium chloride-induced β-Catenin signaling.

(I-Q) Immunostaining for Axin1, the key scaffold of the β-Catenin destruction complex. Note that Wnt signaling relocalizes Axin1 to vesicular structures, and that this effect is strongly enhanced by MITF induction with Tet. For relocalization of other Wnt components (phospho-LRP6, GSK3, and phospho-β-Catenin) after MITF induction and Wnt3a protein treatment, see Figs. 4-S6 to 4-S8.
induction by Tet (Fig. 4-S4). Taken together, these results indicate that MITF enhances Wnt signaling, both in early embryos and in a melanoma setting, in an ESCRT-dependent manner.

**The Destruction Complex Localizes to MITF-induced Vesicles during Wnt Signaling**

To test whether MITF induction affected the relocalization of the β-Catenin destruction complex to endolysosomes/MVBs during Wnt signaling (Taelman et al., 2010; Vinyoles et al., 2014), we first examined the localization of endogenous Axin1, the crucial scaffold protein of this complex, using a monoclonal antibody (Li et al., 2012). In C32 cells, Wnt3a treatment relocalized Axin1 to vesicles (Fig. 4-I-N), and this vesicular localization was greatly enhanced in cells expressing MITF after Tet treatment (Fig. 4-O-Q), which caused an enlargement of the MVB compartment.

MITF-induced vesicles strongly colocalized with endogenous phospho-LRP6 (pLRP6), specifically when C32 cells were treated with Wnt3a (Fig. 4-S5, arrows). This indicated that the activated Wnt receptor complex relocalized into MITF-induced vesicles. In addition, we found that RFP-GSK3 colocalized with CD63-containing vesicles upon Wnt treatment, and that the overlap was particularly prominent in C32 melanoma cells expressing MITF (Fig. 4-S6 A-I, note yellow arrows in panel I’). We also observed that phospho-β-Catenin (p-β-Catenin), a component of the destruction complex (Li et al., 2012) which accumulates in MVBs following Wnt signaling (Taelman et al., 2010), colocalized with the MVB marker CD63 in MITF-expressing C32 cells in the presence of Wnt3a (Fig. 4-S7). The results indicated that the destruction complex components Axin1, GSK3 and p-β-Catenin are translocated to CD63-positive endolysosome/MVBs induced by MITF in the presence of Wnt. The enhanced
sequestration of GSK3 and Axin1 provides a cell biological explanation for how MITF induction increased Wnt signaling (Fig. 4-4F).

**MITF Protein is Stabilized by Wnt**

Wnt signaling has been shown to stabilize many proteins via GSK3-regulated polyubiquitination and degradation (Taelman et al., 2010). This Wnt-induced stabilization of proteins (Wnt/STOP) is emerging as an important branch of canonical Wnt signaling (Acebron et al., 2014). MITF was previously known to be phosphorylated at S409 by p90/RSK1 (Wu et al., 2000), providing a possible priming for the three previously unrecognized carboxy-terminal (S405, S401, S397) putative GSK3 sites identified here (Fig. 4-1A). The priming phosphorylation at S409, in conjunction with another phosphorylation at S73, had been shown to trigger proteasomal degradation of MITF (Wu et al., 2000).

To investigate whether Wnt stabilized MITF protein, we treated C32 melanoma cells (with or without previous induction of MITF expression) with Wnt3a protein (Figs. 4-5A-D). Cycloheximide (CHX) was also added in order to inhibit new protein synthesis. After 5 hours, C32 cells were fixed and immunostained using an anti-MITF monoclonal antibody. Nuclear MITF staining was observed in cells that had been previously induced with Tet. Upon Wnt3a treatment, MITF protein was significantly stabilized (Fig. 4-5 compare panels C and D). Nuclear MITF was quantified and found to be stabilized nearly two-fold by Wnt3a (Fig. 4-5E).

To assess the effect of Wnt3a on endogenous MITF protein, we used the M308 melanoma cell line that expresses MITF from its endogenous promoter at detectable levels. M308 melanoma cells were treated with CHX and control medium, or CHX and Wnt3a, and harvested for western blots at different time points. It was found that Wnt3a treatment prolonged
Fig. 4-5. MITF protein stabilization by Wnt via novel C-terminal GSK3 phosphorylation sites.

(A-D) MITF immunostainings in C32 cells. Wnt3a protein treatment for 5 hours stabilized MITF protein in the presence of cycloheximide (CHX).

(E) Quantification of MITF staining per nucleus from previous experiment (** p < 0.01).
(F) MITF protein levels (normalized to total Erk1/2) from 3 independent western blot experiments upon treatment of M308 melanoma cells with Wnt3a; Wnt prolongs the half-life of endogenous MITF protein.

(G) RT-qPCR of the MITF target gene MART-1 in Tet-induced C32 cells treated with CHX and Wnt3a (** p < 0.01).

(H) RT-qPCR for the MITF target gene x-tyrosinase obtained from *Xenopus laevis* embryos microinjected with *Wnt8, MITF*, or *MITF + Wnt8* mRNAs. *Wnt8* markedly increased MITF activity.

(I) Diagram depicting MITF wild type (MITF-WT) and a MITF GSK3 phosphorylation-resistant mutant (MITF-GM).

(J) Western blot of *Xenopus laevis* embryos injected with equal amounts of mRNA for *MITF-WT* or *MITF-GM* and blotted for MITF. GAPDH was used as a loading control.

(K) Quantification of western blots from three independent *Xenopus* experiments showing that MITF-GM is more stable than MITF-WT.

(L) Diagram of a pMITF<sub>GSK3</sub> antibody raised against the indicated peptide corresponding to the C-terminal region of MITF with two phosphorylations.

(M) pMITF<sub>GSK3</sub> antiserum mirrors the total MITF immunostaining pattern detected with an anti-MITF mAb in Tet-induced C32 cells. This indicates that the phospho-antiserum is specific for MITF.

(N) Western blot of HEK 293T cells transiently transfected with MITF-WT or MITF-GM and blotted for pMITF<sub>GSK3</sub>, total MITF, and GAPDH as a loading control. Note that MITF-GM is not recognized by the phospho-specific MITF antibody.

(O) Western blot of HEK 293T cells transiently transfected with MITF-WT treated with or without BIO, a specific GSK3 inhibitor. Note that pMITF<sub>GSK3</sub>, but not total MITF (anti-MITF mAb), is inhibited by BIO
the endogenous MITF half-life by 45% (Fig. 4-5F). In accordance with this, Wnt3a plus CHX treatment also increased transcript levels of the MITF target gene Melanoma Antigen Recognized by T cells 1 (MART1) (Du et al., 2003) in C32 cells induced to express MITF (Fig. 4-5G), measured by RT-qPCR. In addition, MITF mRNA was significantly more active in promoting the expression of endogenous x-tyrosinase mRNA when co-injected together with Wnt8 mRNA in Xenopus laevis embryos (Fig. 4-5H). Tyrosinase is an MITF target gene that is not normally expressed at the early gastrula stage. Importantly, in early embryos, microinjection of Wnt8 mRNA alone does not increase x-tyrosinase transcripts, suggesting that the strong increase in x-tyrosinase transcripts by MITF in combination with Wnt is mediated by the stabilization and increased activity of the microinjected MITF (Fig. 4-5H). Induction of MITF, as well as Wnt3a treatment, increased cellular proliferation in C32 melanoma cells (Fig. 4-S8).

To test whether the novel putative GSK3 sites on MITF played a role in the stability of MITF, we mutated these Serine residues into Alanine in the human MITF-M (Fig. 4-5I). The resulting MITF GSK3 mutant (MITF-GM) protein was more stable than wild-type MITF (MITF-WT) protein when the same amount of synthetic mRNA was microinjected into Xenopus laevis embryos and analyzed by western blot (Fig. 4-5J). Since equal amounts of mRNA were injected, the effect of the mutations should be posttranscriptional. Quantification of three independent experiments confirmed that MITF-GM was more stable than MITF-WT (Fig. 4-5K). These results indicate that MITF stability and activity are regulated by these novel GSK3 sites.
A Phospho-specific MITF Antibody Demonstrates that the C-Terminal GSK3 Sites are Phosphorylated

To determine whether MITF was phosphorylated at the C-terminal GSK3 sites predicted by the bioinformatics screen, we raised a phospho-specific rabbit antibody directed against the first two GSK3 phosphorylation sites (S401 and S405) adjacent to the S409 priming site (Fig. 4-5L). This anti-pMITF^{GSK3} antibody reproduced the nuclear localization of the anti-MITF monoclonal antibody in melanoma C32 cells upon Tet-driven MITF induction (Fig. 4-5M), indicating that this reagent is specific for MITF and that phosphorylation takes place. Unfortunately, purification in affinity columns failed, but the crude antiserum was effective at 1:5000 dilutions. The pMITF^{GSK3} antibody was phospho-specific, as it did not cross react with the phosphorylation-resistant mutant MITF-GM, while the MITF-WT band was strongly stained in western blots from transiently transfected HEK 293T cells (Fig. 4-5N).

We next tested whether the novel phosphorylation sites in MITF were phosphorylated by GSK3. HEK 293T cells were transiently transfected with MITF-WT and treated the next day with the GSK3 inhibitor BIO for 5 hours. GSK3 inhibition led to the stabilization of total MITF and disappearance of the pMITF^{GSK3} band in western blots (Fig. 4-5O), indicating that the C-terminal phosphorylations of MITF were indeed mediated by GSK3. In Tet-stimulated C32 melanoma cells, MITF was also recognized by pMITF^{GSK3}, and this band disappeared upon GSK3 inhibition with BIO (Fig. 4-S9), suggesting that MITF was phosphorylated at the novel C-terminal GSK3 sites in a melanoma background as well. Taken together, these results indicate that the MITF protein contains three previously unnoticed C-terminal GSK3 phosphorylation sites that regulate its stability.
DISCUSSION

MITF is a melanoma oncogene that is amplified in 30-40% of melanomas (Haq and Fisher, 2011). Other members of the MiT family are also disregulated in cancer, for example TFEB in pediatric renal carcinomas and TFE3 in alveolar soft part sarcomas (Davis and Fisher, 2007; Haq and Fisher, 2011). In this study we uncovered a positive regulatory loop in which Wnt signaling stabilizes MITF independently of de novo protein synthesis. We describe three previously undetected GSK3 phosphorylation sites that are highly conserved in MITF, TFEB, TFE3 and TFEC, and show that these sites in MITF mediate protein stabilization when Wnt inhibits GSK3. We found that high levels of MITF in melanoma cell lines correlated with the expression of a large subset of lysosomal genes. Late endolysosomal structures were greatly expanded when MITF was expressed in the Tet-inducible C32 melanoma cell line. However, these late endolysosomes/MVBs did not form proteolytically active lysosomes in assays using dequenched BSA added to the culture medium. The expansion of late endosomes resulted in increased sensitivity to Wnt signaling through an ESCRT-dependent mechanism. The endosomal/MVB vesicles induced by MITF colocalized with p-LRP6, Axin1, GSK3 and p-β-Catenin in the presence of Wnt, explaining the increased Wnt signaling by the sequestration of the destruction complex in vesicular organelles (Taelman et al., 2010; Dobrowolski et al., 2012; Vinyoles et al., 2014).

The model in Fig. 4-6 proposes that a positive feed-back signaling loop drives the proliferative stages of melanoma. Increased MITF would cause an expansion of late endolysosomal vesicles that potentiates Wnt signaling. In turn, Wnt signaling stabilizes MITF by inhibiting GSK3 and reducing the C-terminal GSK3 phosphorylations of MITF. The proliferative stages of melanoma are associated with a peak in canonical Wnt signaling and also increased
MITF activity (Hoek et al., 2008b). Vesicular trafficking misregulation is emerging as a fundamental hallmark of melanomas and perhaps of other cancers (Alonso-Curbelo, 2014; Ibarrola-Villava, 2014). Our model helps explain how a lineage addiction oncogene could cause perturbations in the endolysosomal pathway and increase cellular responses to Wnt signaling (Fig. 4-6).

**MITF and Lysosomal Biogenesis**

TFEB is the master regulator of lysosomal biogenesis (Sardiello et al., 2009), capable of orchestrating cellular clearance pathways by promoting transcription of a set of genes downstream of the CLEAR element (Palmieri et al., 2011). Recently, it has been shown that TFE3 is capable of a similar response (Martina et al., 2014). Although MITF, TFEB and TFE3 share high sequence homology, MITF had not been previously recognized as being capable of triggering lysosomal biogenesis (Sardiello et al., 2009; Martina et al., 2014). However, the idea that MITF transcription factor may promote a lysosomal gene response is not entirely surprising. One of the MITF E-box consensus DNA binding sites (5′-CACGTG-3′) (Strub et al., 2011) is contained within the TFEB consensus binding site (CLEAR element) (5′-GTCACGTGAC-3′) (Sardiello et al., 2009). In addition, melanomas were recently shown to have a strong enrichment of the lysosome Gene Ontology (GO:0005764) gene set in comparison to other cancers (Alonso-Curbelo et al., 2014).

MITF, the master regulator of melanocytes, regulates the expression of melanosomal genes (Levy et al., 2006). Melanosomes are commonly termed lysosome related organelles (LRO). Although it is tempting to speculate that the correlation between MITF and lysosomal genes described here derives from the relationship between melanosomes and lysosomes,
Fig. 4-6. Model portraying a positive feed-back loop involving MITF, multivesicular bodies (MVBs), and Wnt signaling in proliferative stages of melanoma.

Without Wnt signaling, GSK3 phosphorylates MITF on novel C-terminal phosphorylation sites, targeting MITF for proteasomal degradation. Upon Wnt signaling, destruction complex components are sequestered into MVBs, inhibiting GSK3 and stabilizing MITF. In turn, MITF induces late endolysosomes that further sequester destruction complex components upon Wnt signaling, enhancing overall Wnt responsiveness. This positive feed back loop is proposed to function in the proliferative stages of melanoma, in which MITF and Wnt signaling peak.
melanosomes are distinct from conventional endosomes and lysosomes, and represent a distinct lineage of organelles (Raposo et al., 2001; Raposo and Marks, 2007). Proteomic analyses have revealed that melanosomes contain a unique proteomic profile and share only a few proteins with lysosomes (six in the case of pre-melanosomes, and twelve in the case of mature melanosomes,) (Basrur et al., 2003; Chi et al., 2006). Our analyses revealed that MITF correlates with, and is capable of upregulating, many genes that are exclusively lysosomal and not considered melanosomal. The likely explanation is that MITF, when amplified at the genomic or overexpressed at the transcriptional level in melanomas, may bind promiscuously to TFEB binding sites (CLEAR elements) and drive expression of a large subset of lysosomal genes.

The observation that MITF-M, but not TFEB, significantly correlated with lysosomal genes in melanoma may be a reflection of their distinct regulations. The activity of TFEB is controlled by the mechanistic Target of Rapamycin Complex 1 (mTORC1) (Roczniak-Ferguson et al., 2012; Settembre et al., 2012). In nutrient-rich conditions, TFEB localizes to the lysosomal outer membrane through binding to Rag GTPases (Martina et al., 2014b) via its amino terminal domain. Active mTORC1 on the lysosomal surface phosphorylates TFEB and causes its retention in the cytoplasm by 14-3-3 proteins to (Roczniak-Ferguson et al., 2012; Settembre et al., 2012). Only when mTOR is inhibited, for example during starvation or lysosomal stress, unphosphorylated TFEB is free to enter the nucleus (Roczniak-Ferguson et al., 2012; Settembre et al., 2012). Therefore, TFEB-mediated lysosomal biogenesis depends more on nutritional status and cellular localization than on absolute TFEB levels. The MITF-A and MITF-D isoforms are similarly regulated through their 30 N-terminal amino acids required for binding to Rag GTPases at the lysosome surface (Roczniak-Ferguson et al., 2012; Martina et al., 2014b).

However, the MITF-M isoform, which is the one expressed in melanomas, lacks this N-
terminal domain required for lysosomal localization and mTOR phosphorylation. Consequently, MITF-M is a constitutively nuclear protein (Martina et al., 2014b). We propose that, when overexpressed, MITF-M binds promiscuously to CLEAR element lysosomal genes without being restrained by mTOR signaling. This would be sufficient to drive partial endolysosomal biogenesis, resulting in inactive lysosomes and enhanced Wnt signaling.

Alterations in lysosomal content, distribution, and volume, have been associated with cancer (Kallunki et al., 2012). Recently, it has been shown how melanomas use a lineage-specific Rab7 mediated wiring of the endolysosomal pathway to promote proliferation (Alonso-Curbelo et al., 2014). This is consistent with the very strong increase in Rab7-positive vesicles we found upon MITF induction in C32 cells. It appears that endolysosomal regulation is particularly important in melanoma (Alonso-Curbelo et al., 2014). Perhaps disregulated endolysosomal pathways are a feature of malignancies associated with other MiT family oncogenes as well.

**MITF Enhances Wnt Signaling**

Although MITF induction increased expression of lysosomal genes in melanoma, it did not increase lysosomal activity assessed by the cleavage of BSA-DQ. This was reminiscent of the effects of Chloroquine treatment or Presenilin mutations in which accumulation of late endolysosomal structures enhances Wnt signaling through an increase in the sequestration of the Wnt receptor/β-Catenin destruction complex (Dobrowolski et al., 2012).

Since MITF induction in C32 melanoma cells caused an expansion of MVBs and late endolysosomal/MVB vesicles marked by CD63, LAMP1, and Rab7, we investigated whether
MITF expression could affect Wnt signaling and found that Wnt signaling was enhanced by MITF both in *Xenopus* embryos and in an MITF-inducible melanoma cell line. This increase in Wnt responsiveness required the ESCRT machinery necessary for intraluminal vesicle formation in MVBs. Axin1, GSK3, phospho-β-Catenin and phospho-LRP6 colocalized with MITF-induced vesicles upon Wnt signaling.

Although Wnt is one of the main signaling pathways commonly perturbed in metastatic melanomas (Valsesia et al., 2011), its role in oncogenesis is paradoxical. Canonical Wnt signaling and β-Catenin activation seem to be a key step in the initiation of melanoma (O'Connell and Weeraratna, 2009). However, β-Catenin has also been shown to suppress invasion, and loss of β-Catenin predicts poor rates of survival in patients (Kageshita et al., 2001; Chien et al., 2009; Arozarena et al., 2011). Additionally, BRAF signaling, a hallmark of most melanomas, has been reported to inhibit Wnt signaling in melanoma cells (Biechele et al., 2012). In some melanoma lines, Wnt signaling synergizes with BRAF inhibitors in decreasing tumor growth and increasing apoptosis (Biechele et al., 2012). This has caused re-evaluation of the oncogenic nature of Wnt signaling in these cancers (Lucero et al., 2010). Given the importance of the endolysosomal pathway in Wnt signaling (Taelman et al., 2010; Vinyoles et al., 2014) and Rab7 wiring in melanomas (Alonso-Curbelo et al., 2014), it is tempting to speculate that the sequestration of the destruction complex components, which confers canonical Wnt signaling, is particularly high in melanomas.
MITF Protein Stabilization by Wnt/GSK3

Wnt, through GSK3 inhibition, stabilizes many proteins in addition to β-Catenin (Kim et al., 2009; Taelman et al., 2010; Acebron et al., 2014). Wnt-dependent stabilization of proteins (Wnt/STOP) peaks during G2/M, preventing protein degradation in preparation for cellular division (Bilic et al., 2007). The possibility that Wnt signaling could increase the stability of the MiT family of transcription factors, which behave as oncogenes (Davis and Fisher, 2007; Haq and Fisher, 2011), is potentially significant. We found that Wnt signaling indeed stabilized MITF protein levels in melanoma cell lines. It is well documented that Wnt signaling also participates (through Lef1 and other transcription factors) in driving transcriptional expression of MITF (Dorsky et al., 2000; Takeda et al., 2000; Hsiao and Fisher, 2014). We now find that Wnt can also regulate MITF at the protein degradation level, independently of transcription.

MITF is clearly a driver of melanoma, since a recurrent mutation in MITF predisposes to familial melanoma. This mutant, MITF E318K, has impaired sumoylation and increased activity (Yokoyama et al., 2011). The same mutation also predisposes for renal cell carcinoma (Bertolotto et al., 2011). Ectopic MITF expression in combination with activated BRAF can transform human primary melanocytes (Garraway et al., 2005). We found that Wnt signaling strongly potentiated MITF transcriptional activity, as measured by an increase in MART1 transcripts. Importantly, this experiment was carried out in the presence of Cycloheximide, to prevent de novo synthesis of MITF. During Wnt signaling, only the newly synthesized β-Catenin is competent for signaling (Li et al., 2012). By preventing protein synthesis, we also ruled out any effect of β-Catenin accumulation on the observed increase in MITF activity, indicating that the enhanced activity is due to increased stability, activity, or both. We conclude that Wnt signaling, by inhibiting GSK3, could enhance the stability of MITF and contribute to its
oncogenic effects (Fig. 4-6).

The C-Terminal GSK3 Phosphorylation Sites of MITF

MITF is highly regulated at the transcriptional, posttranscriptional, and posttranslational level (Arnheiter et al., 2007). MITF-M protein has been shown to be phosphorylated at S73, via ERK1/2, and at S409, via p90/RSK1, in response to tyrosine kinase KIT receptor activation (Steingrímsson et al., 2004). MITF has also been proposed to be phosphorylated at S298 by GSK3, and this residue is often mutated in Waardenburg syndrome (Takeda et al., 2000b).

Here are presented three novel GSK3 phosphorylation sites (S397, S401 and S405) conserved in the C-terminus of MITF. When these residues were mutated into alanines, the MITF protein became more stable, suggesting a role for GSK3 in targeting MITF for proteasomal degradation. In agreement with this proposal, the priming phosphorylation at S409 has been shown to participate in MITF proteasomal targeting (Wu et al., 2000). Using a custom anti-phospho MITF\textsuperscript{GSK3} antibody, we confirmed that the new sites are indeed phosphorylated by GSK3 \textit{in vivo}. Phosphopeptides corresponding to the GSK3 phosphorylations have been recorded by proteomic discovery-mode mass spectrometry (see Phosphosite.org), but their regulation has not been previously analyzed in the scientific literature.

MITF, MVBs, Wnt and Melanoma Proliferation

As in the case of Wnt signaling, the role of MITF in melanoma is paradoxical, promoting transcription of genes with antagonistic behaviors (Palmieri et al., 2009). As a melanocyte master
regulator, MITF drives cells towards differentiation, and high MITF levels have antiproliferative effects (Wellbrock and Marais, 2005). However, MITF has been also described as a lineage addiction, or lineage survival, oncogene required for melanoma survival and proliferation (Steingrímsson et al., 2004; Garraway et al., 2005; Garraway and Lander, 2013). The levels of MITF protein are critical in melanomas, as they determine cellular phenotype: low levels cause G1 arrest, stem cell-like properties, and often confer invasive behavior (Carreira et al., 2006). Intermediate MITF levels endow cells with increased proliferative capacity. However, higher levels drive cells toward differentiation and G1 arrest. Thus, MITF is considered a molecular rheostat (Carreira et al., 2006; Hoek and Goding, 2010, Strub et al., 2011; Cheli et al., 2011).

Melanomas, in terms of their expression profiles and clinical behavior, can be subdivided into two phenotypes: proliferative and invasive. Among the genes upregulated in the proliferative melanoma state are many MITF targets as well as many Wnt target genes (Hoek et al., 2008b). This contrasts to the invasive state, in which MITF and Wnt targets are downregulated (Hoek et al., 2008b). These different cellular phenotypes coexist within a heterogeneous tumor, and are determined by MITF levels (Hoek et al., 2008b). However, melanoma cells have the capability to switch phenotypes by modifying MITF levels. Recently, this phenotype switch has been elegantly exploited as part of an effective antimelanoma therapy strategy (Sáez-Ayala et al., 2013).

The results presented here suggest that in the proliferative stages of melanoma increased MITF levels may potentiate Wnt signaling by expanding the endolysosomal compartment, and that Wnt in turn stabilizes MITF by preventing GSK3 mediated proteasomal degradation.
MATERIALS AND METHODS

Analysis of gene expression by microarray

Melanoma cell lines were cultured in RPMI medium (Life Technologies) supplemented with penicillin/streptomycin and fetal bovine serum (FBS). All 51 cell line cultures used for arrays were harvested at 50-70% confluency, centrifuged, quick frozen, and characterized by mtDNA sequence prior to use. For microarray analysis of gene expression, the RNA was isolated using the QIAGEN RNeasy protocol and quantitated using a NanoDrop Spectrophotometer (Agilent Technologies). Eight hundred twenty five nanograms of high-quality total RNA with RIN (RNA integrity number) greater than 8.0 was labeled with cyanine 5-CTP or cyanine 3-CTP using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) and purified on RNeasy Mini columns (QIAGEN). Labeled RNA was then hybridized to Agilent Human 44K expression arrays that include 44,000 probes and compared with a labeled mixed reference sample consisting of a pool of equal amounts of RNA from each of 47 melanoma cell lines. Analysis of the microarray data was done using Rosetta Resolver (v. 7.2.2.0) system with p values equal or less than 0.01.

Gene Set Enrichment Analysis (GSEA)

The correlation between MITF or TFEB and the set of lysosomal genes was analyzed by the gene set enrichment analysis approach (Subramanian et al., 2005). The set of 89 lysosomal genes was from Sardiello et al. (Sardiello et al., 2009). Normalized gene expression data for
melanoma cell lines was obtained from the Hoek et al. dataset (Hoek et al., 2008), which consists of three independent subsets: Zurich, Mannheim, and Philadelphia, containing among them 83 additional melanoma lines. Each subset was scaled and the Pearson correlation between MITF or TFEB and all genes was calculated. MITF was represented as a single probe and for TFEB the average scaled expression vector of two probes was used. Each gene was collapsed to its probe with the highest absolute correlation. Genes were ranked by their correlation with MITF or TFEB and the three subsets combined using average gene ranks. The combined ranked lists were analyzed for enrichment of the lysosomal gene set. Statistical significance was assessed with a permutation based Kolmogorov-Smirnoff (KS) non-parametric rank test (1000 permutations) (Subramanian et al., 2005).

Cell culture

For MITF induction in the C32 MITF Tet-inducible melanoma line, cells were grown in medium containing Tetracycline hydrochloride 1 μg/ml (Tet) (Sigma) for 4 days to allow MITF protein to be synthesized. Recombinant murine Wnt3a protein (PeproTech) was added to C32 cells at 80 ng/ml for 5-6 h. Chloroquine diphosphate (CQ) was dissolved in water and added to C32 cells at 100 μM (Sigma). For GSK3 inhibition, cells were treated with BIO (or meBIO as a control) (Meijer et al., 2003) at 5 μM for 5 h or LiCl at 30 mM for 8 h. For inhibition of protein synthesis, Cycloheximide (Sigma) was dissolved in ethanol and used at a final concentration of 20 mg/ml (Taelman et al., 2010).
Immunostainings

For immunostainings, round coverslips were placed in 12-well plates and extensively washed with ethanol, Dulbecco’s phosphate buffered saline (DPBS, Gibco), and culture medium. C32 melanoma cells, previously grown with or without Tet for four days, were seeded into these 12-well plates, and 24 h later treated with Wnt3a. After 6 h of Wnt3a treatment, cells were fixed with fresh 4% paraformaldehyde (Sigma #P6148) (PFA) in DPBS for 20 min, washed for 15-20 minutes three times in freshly prepared DPBS, permeabilized by treatment with 0.2% Triton X-100 in DPBS for 10 min, washed for 15-20 minutes three times in DPBS and treated with 0.5% SDS for 5 min for antigen retrieval. Wells were then washed for 15-20 minutes three times in DPBS and blocked with 5% goat serum plus 0.5% BSA in DPBS for at least 2 hours (blocking solution). Cells were then incubated with primary antibodies, diluted 1:4 in blocking solution, overnight at 4°C. The following day, wells were washed for 15-20 minutes three times DPBS, and secondary antibodies (diluted in 1:4 blocking solution) applied for two hours at room temperature. After washing in DPBS, coverslips were removed and mounted in ProLong Gold antifade reagent with DAPI (Life Technologies) to stain cell nuclei. A Zeiss Imager Z.1 microscope with Apotome was used to analyze and photograph immunofluorescence. To quantify immunostainings for MITF protein in C32 cells, staining of individual nuclei was measured with ImagJ software (NIH). Since uninduced C32 cells lack MITF staining, the perimeter of the nucleus was determined by DAPI staining. The MITF protein levels of 120-150 individual nuclei were measured for each condition. Primary antibodies used for immunostaining in this study were: anti-MITF 1:250 (DAKO #M3621), anti-phospho-β-Catenin 1:300 (S33/37/T41) (Cell Signaling Technologies #9561), anti-Axin1 clone A5 1:300 (Millipore #05-1579), anti-phospho-LRP6 1:300 (S1490) (Cell Signaling Technologies #2568S), anti-CD63
1:400 (BD Pharmingen #556019), anti-Rab7 1:400 (D95F2) (Cell Signaling Technologies #9367S), and anti-LAMP1 1:400 (DSHB #H4A3). For generation of the custom pMITF\textsuperscript{GSK3} antibody, a synthetic peptide [Ac-C(Ahx)PGA(pS)KTS(pS)RRS-amide] was used to immunize a rabbit (Covance). Although affinity purification failed, the remaining high titer antiserum is effective at a concentration of 1:250 for detecting pMITF\textsuperscript{GSK3} in immunostainings of C32 melanoma cells. Secondary antibodies were Alexa Fluor 488-conjugated AffiniPure Donkey Anti-Rabbit IgG and Cy3 conjugated AffiniPure Donkey anti-Mouse IgG (Jackson ImmunoResearch Laboratories).

BSA-DQ Lysosomal Activity Assay and Lysotracker on living cells

For studying lysosomal proteolysis on endocytosed BSA, C32 cells previously grown with or without Tet, were plated in 6-well plates. Upon 60% confluency, cells were treated with 5 µg/ml of BSA-DQ (DQ\textsuperscript{TM} Red BSA, Molecular Probes, #D-12051) diluted in prewarmed medium and incubated at 37°C for 6 hs. Cells were then briefly washed twice with prewarmed DPBS, trypsinized, fixed in suspension with 4% PFA for 15 min at room temperature, and washed extensively with DPBS at room temperature. Fluorescence of cleaved BSA-DQ was analyzed by flow cytometry. For visualizing LysoTracker stainings by microscopy, C32 cells were previously grown with or without Tet were plated in 12-well plates containing coverslips as described above for immunostainings. Upon 60% confluency, cells were treated with Lysotracker (LysoTracker Red DND-99, Invitrogen, #L7528), diluted 1:1000 in prewarmed medium, incubated for 1 min at 37°C, and washed with prewarmed DPBS. Cells were fixed with
4% PFA for 15 min at room temperature in the dark, and washed extensively with DPBS. Coverslips were then mounted with ProLong Gold antifade reagent with DAPI (Life Technologies) and visualized with Zeiss Imager Z.1 microscope with Apotome. For quantitatively analyzing Lysotracker stainings, C32 cells were previously grown with or without Tet were plated in 6-well plates and, upon 60% confluence, cells were treated with Lysotracker as described above in prewarmed medium and incubated for 1 min at 37°C, and washed extensively with prewarmed DPBS. Cells were then trypsinized, fixed in suspension with 4% PFA for 15 min at room temperature in the dark, washed several times with DPBS, and analyzed by flow cytometry.

**DNA constructs**

Human MITF-M (GB# NM000248) was cloned into the pCS2 vector (gift from Dr. D. Turner). This MITF-M construct was used to generate the MITF GSK3 mutant (MITF-GM) by PCR-based site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis, Stratagene). RFP-GSK3 was from Taelman et al., 2010. The CLEAR element Luciferase construct was from (Sardiello et al., 2009), and was transfected at 0.6 µg per 12-well plate, together with 0.2 µg of SV40-Renilla luciferase construct for normalization (Taelman et al., 2010).

**Cell Culture**

HEK 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 1% glutamine, and 1% Pen/Strep. All melanoma cells
were cultured in RPMI 1640 (with L-glutamine) (Life Technologies) containing 10% Tetracycline-free FBS (Omega Scientific), 1% Pen/Strep. For C32 melanoma cells, 0.5 µg/ml Blasticidin and 100 µg/ml Zeocin were added in order to maintain selection of the Tet-inducible MITF. All cells were cultured at 37°C in 5% CO₂.

**Xenopus laevis embryo assays**

For *Xenopus* mRNA microinjections, mRNAs were synthesized with mMessage mMachinie SP6 (Ambion). For Wnt signaling assays, 20 pg of the *TCF SuperTopFlash luciferase* (Veeman et al., 2003) reporter were coinjected with *Wnt8* (2 pg), *MITF* (100 pg), HRS morpholino (4 nl of 0.3 mM HRS MO), or *Vps4EQ* mRNA (500 pg) were co-injected. Equal amounts of total mRNA were injected in all samples by adding *GFP* mRNA. For embryo Wnt signaling assays through *chordin in situ* hybridizations, suboptimal amounts of *Wnt8* mRNA (0.1 pg) were injected with or without the addition of *MITF-M* mRNA (320 pg). For analyzing the effect of the novel putative GSK3 sites in MITF on protein stability, *Xenopus* embryos were microinjected four times at the 4-cell stage with 80 pg of *MITF-WT* or *MITF-GM* mRNA, and lysed at stage 13 with lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and Protease inhibitor #10863600 from Roche). *Xenopus* whole-mount *in situ* hybridizations were carried out according to the protocol described at [http://www.hhmi.ucla.edu/derobertis/index.html](http://www.hhmi.ucla.edu/derobertis/index.html).

**Knockdown experiments, DNA transfections, reverse transfection, and lentiviral transductions**

For HEK 293T cells, DNA constructs were transfected with BioT (Bioland Scientific) 24
hours after plating cells. In C32 melanoma cells, RFP-GSK3 DNA was transiently incorporated by reverse transfection with Lipofectamine 2000 following the reverse transfection protocol from Invitrogen. For knockdown of HRS in C32 cells, siRNAs targeting human HRS were ON-TARGETplus SMARTpool siRNAs (L-016835) and the control siRNA pool (D-001810) were from Thermo Scientific. Cells were reverse transfected with siRNA 24 hours prior to Wnt3a treatment. siRNAs were transfected using Lipofectamine 2000 and following the reverse transfection protocol from Invitrogen. For Wnt signaling assays, a β-Catenin activated reporter (BAR)-Firefly Luciferase reporter lentivirus containing Puromycin selection marker (Biechele and Moon., 2008) was transduced into C32 MITF-inducible melanoma cells. After selection with Puromycin, the C32-BAR cells were then transduced with an Ef1α-Renilla lentivirus (Biechele and Moon., 2008) for normalization purposes. For transductions, 1 ml of lentivirus (0.7 μg/ml) was incubated in a 6-well plate at 70% confluency for 16 hours. After incubation, medium was replaced and 24 hs later cells were trypsinized. One week after transduction, cells were selected with Puromycin.

**Flow Cytometric Measurement of Immunostainings**

To quantitate immunostaining intensities by flow cytometry, two 10 cm plates of cells per condition were grown to 70% confluency, trypsinized, and collected in a 15 ml conical. Cells were gently spun, supernatant removed, and fixed in suspension in 4% PFA in Eppendorf tubes on an end-over-end rotator at room temperature for 20 minutes. Cells were then spun at 1400 rpm in a table-top centrifuge, supernatant removed, resuspended in freshly prepared DPBS, and rotated in an end-over-end rotator at room temperature for 20 minutes. This DPBS washing step was repeated three times. Cells were then permeabilized by resuspending them in 0.2% Triton X-
100 in DPBS (Gibco), and left rotating for 10 min. Cells were then washed two times in DPBS following the method described above. For antigen retrieval, cells were incubated in 0.5% SDS for 5 min, and washed three times in DPBS. For blocking, cells were resuspended in 5% goat serum with 0.5% BSA in DPBS (blocking solution) for at least 2 hours. Cells were then spun down and resuspended with primary antibodies, diluted in 1:4 blocking solution, and left rotating overnight at 4°C. The following day, cells were washed three times in DPBS as before. Cells were resuspended in secondary antibodies diluted in 1:4 blocking solution and rotated for two hours at room temperature in the dark. The three subsequent washes in DPBS were also performed in the dark. Fluorescence was measured by flow cytometry for 10000 cells counted. Antibodies used were: anti-CD63 1:400 (BD Pharmingen #556019), anti-LAMP1 1:400 (DSHB #H4A3). Secondary antibodies were Alexa Fluor 488-conjugated AffiniPure Donkey Anti-Rabbit IgG and Cy3 conjugated AffiniPure Donkey anti-Mouse IgG (Jackson Immuno Research Laboratories).

**Flow Cytometry**

Quantification of fluorescence by flow cytometry was performed in an LSRII flow cytometer (Becton Dickinson). 10000 events per sample were collected by FACSDiva Version 6.0 and analyzed by CellQuest software.

**Western Blot Analyses**

For western blot analyses, cells were cultured in 6-well (melanoma cells) or 12-well (HEK 293T cells) plates. Cells were lysed in radioimmunoprecipitation assay buffer (RIPA lysis buffer, 0.1% NP40, 20 mM Tris/HCl pH 8, 10% Glycerol) supplemented with protease inhibitors
(Roche #04693132001) and phosphatase inhibitors (Calbiochem 524629). Western blots were performed using standard procedures. Odyssey™ Blocking Buffer (LI-COR) diluted in PBS (1:1 ratio) was first used to block nitrocellulose membranes for one hour at room temperature. All antibodies were diluted in PBS:Odyssey™ Blocking Buffer supplemented with 0.1% Tween 20. Primary antibodies, anti-MITF (DAKO #M3621) (diluted 1:1000), anti-pMITF\textsuperscript{GSK3} serum (diluted 1:5000), and anti-GAPDH (Cell Signaling Technologies #2118S) (14C10) (diluted 1:7000), were incubated overnight at 4°C. Membranes were then extensively washed with Tris-buffered saline Tween 20 (TBST buffer) and incubated with fluorescently labeled secondary antibodies for two hours at room temperature. Fluorescently labeled secondary antibodies used were IRDye 800CW Donkey anti-Rabbit immunoglobulin G (IgG) (LI-COR Biosciences 926-32213; 1:5,000) and IRDye 680RD Donkey anti-Mouse IgG (LI-COR Biosciences 926-68072; 1:5,000). An Odyssey 9120 infrared imaging system (LI-COR) was used to acquire images.

**Cell Viability Assays**

C32 cells were plated in opaque 96 well-plates at 3000 cells/well density. Cells were treated in several replicates (8 wells per condition) with 4 different conditions: ethanol, Wnt3a at 1:500 for 3 days, tetracycline at 1:5000 for 5 days and combination of tetracycline at 1:5000 for 5 days with Wnt3a at 1:500 for 3 days. After incubation for 120 hours total, cell viability was determined using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI), an ATP-based bioluminescent assay, as per manufacturer’s instructions. Each experiment was repeated independently at least 3 times.

**Luciferase Assays**
For Luciferase measurements of cell lysates, cells from 12-well plates were lysed with 180 μl of Passive Lysis Buffer (Promega). For luciferase measurements of *Xenopus* ectodermal explant lysates, embryos were co-injected four times with the corresponding mRNAs together with 20 pg of the *TCF SuperTopFlash* reporter (see supplemental references) at 4-cell stage. Ectodermal explants were excised at mid-blastula (stage 8.5). Thirty ectodermal explants were harvested per condition and lysed in 100 μl of Passive Lysis Buffer (Promega). Lysates were then spun at maximum speed in a tabletop centrifuge at 4°C, and luciferase assays were performed on the supernatant with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions, using a Glomax Luminometer (Promega).

RT-qPCR

For quantitatively analyzing the expression of endogenous mRNA transcripts, total RNA was extracted from cultured cells (one 6-well plate per condition) or *Xenopus* embryos (seven stage 13 embryos per condition), using Absolutely RNA Microprep Kit (Agilent Technologies). cDNA synthesis was carried out using AffinityScript™ Multi Temp Reverse Transcriptase (Agilent Technologies) and mRNA levels analyzed using the SYBR green reagent with ROX as reference dye. Relative gene expression levels were determined using the Comparative C_t method using the housekeeping gene GAPDH in case of cultured cells, or *Ornithine Decarboxylase* (*xODC*) in case of *Xenopus* samples, for normalization. Primers for lysosomal genes and negative controls were those described by Sardiello et al., 2009 (Sardiello et al., 2009). RT-qPCR conditions and primers for *xODC* can be found at http://www.hhmi.ucla.edu/derobertis/protocol_page/protocol.html. RT-qPCR primers for *x-tyrosinase* were: Fwd: GAAACGTTGACTTTGCCCAT and Rev:
CTGCAGACAATCTCCCATGA, and for MART1: Fwd: AGATGCAAGAGAAGATCTC, and Rev: GCTCTTAAGGTGAATAAGGTGG.

**Statistical analyses**

Results from three or more independent experiments are presented as the mean ± SEM. Excel (Microsoft) was employed for statistical analyses, applying the two-tailed t test as appropriate. Significant differences of means are indicated as ** < 0.01 and *** < 0.001. For Xenopus embryo assays, western blot analyses, siRNA in tissue culture, lentiviral transductions, luciferase assays, RT-qPCR analysis, and additional cell culture information, see Supplemental Materials and Methods.

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Fig. 4-S1. *MITF* expression correlates with the expression of many lysosomal genes containing a CLEAR (Coordinated Lysosomal Expression And Regulation) promoter element in melanoma cells.

Microarray analysis shows that 51 melanoma cell lines clustered into two distinct groups according to their expression signature of 63 CLEAR element lysosomal genes (Sardiello et al., 2009). One group had high *MITF* expression (in red) and the other had low *MITF* expression (in blue). The melanoma group with high *MITF* expression upregulated many, but not all, lysosomal genes containing a CLEAR element in their promoter. Horizontal lines in this heat map correspond to individual cell lines, with lysosomal gene expression shown vertically. *MITF* expression was added as a reference, for it is not a lysosomal gene. The cell lines were allowed to sort according to their lysosomal gene expression signature. Surprisingly, all cell lines with high *MITF* mRNA expression (and *MITF* amplification) clustered as a high lysosomal gene expressing group.
HEK 293T cells:

**Fig. 4-S2.** Transient transfection of MITF into HEK 293T cells increased transcription of many CLEAR element lysosomal genes, as measured by RT-qPCR. 

MITF transcription increased in MITF-transfected 293T cells, as expected. MITF transient transfection upregulated transcription of the CLEAR element lysosomal genes cathepsin A (*CTSA*), mucolipin 1 (*MCOLN1*), prosaposin (*PSAP*), N-acetylglucosamine-6-sulfatase (*GNS*), serine carboxypeptidase 1 (*SCPEP1*), Sialidase 1 (*NEU1*), α-galactosidase (*GLA*), and TFEB. Transcription of chloride channel voltage sensitive 7 (*CLCN7*) remained unchanged, while it was decreased for arylsulfatase A (*ARSA*), by MITF transfection in HEK 293T cells. As a negative control, MITF transfection did not upregulate transcription of the F-box protein 11 (*FBXO11*). Error bars indicate the standard error of the mean from three independent experiments.
Fig. 4-S3. MITF-induction increases immunostaining of the late endosomal marker Rab7, in MITF-induced vesicles in C32 melanoma cells.

(A-B) Immunostainings for Rab7 in uninduced C32 melanoma cells.

(C-D) MITF-induced C32 melanoma cells show a striking increase in Rab7 levels. (B’-B’’ and D’-D’’). Note that Rab7 localizes specifically to vesicles that are visible by light microscopy. Upon MITF induction, cells undergo a great expansion of these late endolysosomes marked with Rab7.
Fig. 4-S4. MITF and Chloroquine (CQ) enhance Wnt signaling in melanoma cells.
In uninduced C32 cells, CQ (100 μM) enhances Wnt3a signaling nearly 2-fold. Similarly, MITF induction by Tet addition enhanced signaling by Wnt3a (**P < 0.001). Signaling was assayed using the Wnt BAR-reporter (see methods) and normalized to Renilla under the EF1α promoter control.
Fig. 4-S5. Activated Wnt receptor phospho-LRP6 (p-LRP6) localizes to MITF-induced late endolysosomal vesicles when cells are treated with Wnt3a protein.

(A-F’’) Wnt3a induces phosphorylation of LRP6. Endogenous p-LRP6 appeared as punctae with vesicular localization, marking active Wnt signalosomes/MVBs by immunostaining (Taelman et al., 2010, 8).

(G-I’’) Induction of MITF with Tet increased the number of vesicles, but without Wnt stimulation, these vesicles were devoid of p-LRP6 (arrows).

(J-L’’) Upon Wnt3a treatment, MITF-induced vesicles strongly accumulate p-LRP6 (arrows), indicating that the MITF-induced MVB/late endolysosomes participate in Wnt signaling. Note that MITF-induced vesicles contain p-LRP6 only when C32 cells were treated with Wnt3a (arrows).
Fig. 4-S6. MITF induction increases the colocalization of Wnt-induced GSK3 puncta and CD63 positive MVBs in C32 cells transfected with RFP-GSK3.

(A-F’) Wnt3a treatment increases the number of GSK3-RFP puncta, which sometimes colocalize with endogenous CD63, a marker enriched in the intraluminal vesicles of MVBs.

(G-I’) Wnt increases the colocalization of GSK3-RFP puncta together with endogenous CD63 positive vesicles in cells induced to express MITF. In these cells, RFP-GSK3 puncta are frequently seen enveloped in CD63 positive structures (arrows).
Fig. 4-S7. MITF-induced CD63-positive MVBs colocalized with the Wnt signalosome component p-β-Catenin after Wnt treatment in C32 melanoma cells.

(A-H’) Wnt3a signaling increases p-β-Catenin, which localizes to MVBs marked by CD63. (I-L’) MITF induction causes a further increase in p-β-Catenin, which is localized in CD63 positive MVBs. Note that MITF induction caused a strong expansion of CD63 positive MVBs compartment (compare G and K), and that the increased colocalization of p-β-Catenin and CD63 upon Wnt signaling in MITF induced cells resides in vesicles visible through DIC microscopy (I, L, L’).
Fig. 4-S8. Wnt3a and MITF increase C32 melanoma proliferation in ATP-based cell viability assays.

Wnt3a treatment for 3 days, as well as MITF induction for 5 days, significantly enhanced C32 cell proliferation. Combination of MITF induction and Wnt3a further increased C32 cell growth.
Fig. 4-S9. The novel C-terminal GSK3-regulated phosphorylations take place in MITF in C32 melanoma cells, as monitored by a phospho-specific antibody. In Tet-induced C32 cells, a band corresponding to pMITF\textsuperscript{GSK3} was detected, which disappeared upon treatment with the GSK3 inhibitor BIO.
CHAPTER 5

Conclusions and Perspectives
This concluding chapter consists of two parts. First, I will discuss the implications of the Crescent and Sizzled as Tolloid inhibitors for the self-organizing BMP/Chordin gradient that determines dorsal-ventral (DV) patterning. Second, I will address my investigations on the mechanism of protein stabilization by Wnt/GSK3 signaling. In particular, I will review the perspectives of my work on the regulation of MITF, a member of the MiT family of oncogenic transcription factors, by GSK3 phosphorylations and its role in endolysosomal biogenesis and Wnt signaling.

Creating a Resilient Gradient:

During gastrulation, two main regulatory centers are formed: The Spemann organizer (dorsal center) and the ventral center. Transplantation experiments demonstrated the inductive properties of the Spemann organizer (Spemann and Mangold, 1924) and, more recently, the ventral center has also been shown to play an equally crucial role in the establishment of dorsal and ventral fates (De Robertis and Kuroda, 2004). These two evolutionarily conserved signaling centers act and react to each other’s signals, creating a robust gradient of signaling that determines DV polarity.

Determining a Dorsal Pole

In *Xenopus*, this asymmetry originates at fertilization upon sperm entry into the oocyte. This event triggers the rotation of the cortex (cortical rotation) and the movement of dorsal determinants towards the future dorsal pole, in a process that requires microtubules (Gerhart et al., 1989; De Robertis et al., 2000; Weaver and Kimelman, 2004; Houston, 2012). Although the
exact molecular nature of these dorsal determinants is one of the greatest mysteries of developmental biology, they are thought to be comprised of vegetally deposited mRNAs, proteins and organelles. Furthermore, dorsal determinants have been shown to activate canonical Wnt signaling, with stabilization of β-Catenin on the dorsal side (Schneider et al., 1996; Larabell et al., 1997; Heasman, 2006). This dorsal Wnt signaling activates the transcription of a gene network that will give rise to the Spemann organizer after the midblastula transition (Bier and De Robertis, 2015). Altering the cortical rotation and translocation of dorsal determinants, for example centrifugation or UV irradiation, disrupts normal DV patterning (Black and Gerhart, 1985; Vincent and Gerhart, 1987).

**The Signaling Centers Secrete Molecules of Relater Structure that Confer Resilience to the BMP Gradient**

During gastrulation, the Spemann organizer and the ventral center are formed. These two signaling centers, localized at opposite poles of the developing embryo, are in charge of producing and maintaining a BMP morphogenetic signaling gradient that patterns the early embryo and determines dorsal and ventral fates (De Robertis, 2009). These gastrula signaling centers have an opposing yet parallel relationship. The ventral center functions as the main source of BMP growth factors, while the Spemann organizer is specialized in secreting growth factor antagonists, for example the BMP inhibitors Chordin, Noggin, and Cerberus (De Robertis and Kuroda, 2004). The ventral center also expresses Wnt-8, whereas the dorsal center secretes the Wnt antagonists Dkk, Crescent, and sFRP2 (Christian et al., 1991; Glinka et al., 1998; Pera and De Robertis, 2000). Chordin, a key morphogen in the formation of the BMP gradient is cleaved by Tolloid metalloproteinases (Piccolo et al., 1997). There are three Tolloids expressed
in the early *Xenopus* gastrula, Xolloid (Xld), Xolloid-related (Xlr) and BMP1 (Goodman et al., 1998; Dale et al., 2002). The cleavage of Chordin by these proteases is facilitated by Ont-1, a scaffold that brings enzyme and substrate together (Inomata et al., 2008).

The BMP signaling gradient generated by the Spemann organizer and ventral center is capable of patterning the three germ layers, endoderm, mesoderm, and ectoderm in a concerted fashion. In order to accomplish this, it has been proposed that cells from both ectoderm and endomesoderm read their positional information from a single BMP signaling gradient, located in the Brachet’s cleft, a narrow space that separates both germ layers. This crevice, which contains extracellular matrix, functions as a highway for diffusion of Chordin and BMPs, and conceivably other secreted factors over long distances in the gastrulating embryo (Plouhinec et al., 2013).

BMP signaling stems from ventral regions, promoted by the ventral center, and is inhibited dorsally by extracellular proteins secreted by the Spemann organizer. This opposing nature of the signaling centers is the key driver behind the formation of the BMP signaling gradient that will pattern dorsal and ventral tissues (De Robertis et al., 2009; Bier and De Robertis 2015). However, both dorsal and ventral centers show striking similarities with regards to their repertoire of secreted factors. For many proteins secreted in one organizing center, there is another homologous protein secreted on the opposite pole (Fig 5-1) (Reversade and De Robertis, 2005; Ambrosio et al., 2008; Inomata et al., 2008; Ploper et al., 2011). For example, the ventral center expresses BMP4/7, while the dorsal center secretes ADMP/BMP2 (Reversade and De Robertis, 2005). Likewise, the dorsally secreted BMP antagonist Chordin has a ventrally expressed counterpart, Crossveinless 2 (CV2; Ambrosio et al., 2008). In addition, the dorsal center produces the sFRP Crescent, while the ventral center secretes Sizzled, a closely related sFRP (Lee et al., 2006; Ploper et al., 2011) (Fig. 5-1).
Fig. 5-1. *Xenopus* DV patterning is regulated by the interaction of a dorsal and a ventral Signaling Center. These signaling centers create and maintain a gradient of BMP signaling from ventral to dorsal. In order to achieve this, they secrete proteins that interact with each other in the extracellular space, forming a network that generates and preserves the BMP signaling gradient. Many components of this signaling network have counterparts of similar structure and function secreted on the opposite pole (Sizzled/Crescent, CV2/Chordin, BMP4/ADMP, BMP7/BMP2), and which are under opposite transcriptional control. Ventral center genes (Sizzled, CV2, BMP4, BMP7, etc) are transcriptionally activated by high BMP, while dorsal center genes (Crescent, Chordin, ADMP, BMP2, etc) are expressed in areas of low BMP signaling.
Self-regulation is a fundamental property of the developing gastrula (Spemann, 1938). Evolution has exquisitely selected for molecular pathways and circuits that robustly maintain the BMP signaling gradient and fine-tune signaling inputs and responses in order to consistently achieve proper tissue patterning (Bier and De Robertis, 2015). In order to generate and maintain this BMP gradient, key genes secreted by dorsal and ventral signaling centers are subjected to inverse transcriptional regulation.

High BMP signaling activates the expression of ventral genes while inhibiting the expression of dorsal genes (Reversade and De Robertis, 2015). Accordingly, dorsal genes are transcriptionally activated in regions of low BMP (Niehrs and Pollet, 1999; Pollet et al., 2005). This positive feed-back loop enhances BMP signaling in ventral regions and inhibits it dorsally. However, since the developing gastrula secretes homologous proteins on each side, “pro-BMP” factors (BMP2/ADMP) are also secreted dorsally and “anti-BMP” factors (Sizzled, Bambi) are secreted ventrally. Although this might seem counterintuitive, the ventral expression of anti-BMP factors and the expression of dorsal BMPs serve an important regulatory role by limiting the activity of each signaling center through the creation of negative feedback loops, which equilibrate BMP signaling upon perturbations of the system. For example, when BMP signaling reaches its lowest point on the dorsal pole, ADMP/BMP2 are secreted dorsally by the Spemann organizer in order to compensate for the lack of BMP signaling, and finely tune down Spemann organizer genes (Reversade and De Robertis., 2005). These BMPs are rapidly bound by Chordin in the extracellular space, and these complexes have been proposed to diffuse ventrally where, after being liberated by Tollooids, they can transduce BMP signaling (Zakin and De Robertis., 2010). An extreme example of this is observed during dorsal-ventral bisections, where ADMP and BMP2 compensate for the loss of ventral BMPs and endows the dorsal half with the BMP signal.
morphogens required for proper DV patterning (Reversade et al., 2005). On the ventral side, this type of negative feedback regulation is illustrated by Sizzled, the ventrally expressed sFRP that acts in an anti-BMP fashion by inhibiting Tolloid metalloproteinases that degrade Chordin, the dorsally expressed BMP antagonist that plays a critical role as a morphogen (Lee et al., 2006). High BMP signaling drives Sizzled expression, which will lead to increased Chordin levels, resulting in subsequent BMP inhibition and completing the feedback loop (Yabe et al., 2003; Lee et al., 2006). The existence of these homologous protein pairs expressed on opposite poles of the developing gastrula confers robustness to the signaling network.

Crescent and Sizzled Balance Tolloid Protease Activity from Opposite Poles

When Sizzled was demonstrated to regulate DV patterning by competitively inhibiting Tolloid metalloproteinases (Lee et al., 2006; Muraoka et al., 2006), it was unclear whether Crescent, the dorsally expressed counterpart of Sizzled, also functioned in this manner. Although Sizzled contains a Frizzled domain, it is unable to bind Wnt proteins and modulate Wnt signaling (Collavin and Kirschner, 2003; Yabe et al., 2003). Crescent, however, similarly to other sFRPs, does bind Wnt ligands. Crescent regulates canonical Wnt signaling by binding Wnt ligands in the extracellular space, thus outcompeting Frizzled receptors on the plasma membrane of Wnt-sensing cells (Shibata et al., 2005; Mii and Taira, 2009; Dickinson and Sive, 2009). Crescent also regulates the non-canonical Wnt pathway, by binding to Wnt11 and Wnt5a, modulating convergent extension movements during gastrulation and neurulation (Shibata et al., 2005). Additionally, Crescent has been shown to have a positive role during Wnt signaling by facilitating the diffusion of Wnt proteins and allowing Wnt signals to expand significantly, reaching cells and tissues far beyond the Wnt source (Mi and Taira., 2009).
SFRPs are highly expressed in the *Xenopus* gastrula. During a screen for cDNAs that encode secreted proteins, a surprising 24% of isolates were found to represent sFRPs (Pera and De Robertis., 2000). Since the Spemann organizer secretes a cocktail of Wnt inhibitors such as Dkk, Cerberus, Frzb, and sFRP2, the function of Crescent in the organizer was thought to be that of yet another Wnt inhibitor (Pera and De Robertis., 2000; Shibata et al., 2005; Mii and Taira., 2009). However, due to its structural similarity with Sizzled, and of the emerging concept that homologous proteins pairs of similar structure and function are secreted from both dorsal and ventral signaling centers, a working hypothesis was investigated in which Crescent might function in DV patterning by inhibiting Tolloid proteinases that degrade Chordin, thereby regulating the BMP signaling gradient (Ploper et al., 2011).

Using a combination of biochemical and embryological techniques, Crescent was shown to bind the Tolloid enzyme BMP1 with high affinity and to competitively inhibit Tolloid activity in vitro by enzyme kinetics assays. Crescent mRNA overexpression and protein injections resulted in dorsalized phenotypes that, importantly, require Chordin. Additionally, when the homologous mutation that renders Sizzled inactive as a Tolloid inhibitor was introduced in Crescent, the Tolloid inhibitory effect of Crescent was completely abolished, and the anti-BMP phenotypes of *Xenopus* embryos were greatly reduced. This work placed Crescent in the Chordin/BMP pathway that regulates DV patterning in the developing gastrula by protecting Chordin from the proteolytic activity of Tolloid metalloproteinases (Ploper et al., 2011). A compensatory mechanism was proposed in which Crescent and Sizzled act in a seesaw manner: dorsal and ventral inhibitors of Tolloids metalloproteinases can adjust the BMP gradient by fine-tuning the proteolytic degradation of Chordin in order to achieve proper patterning (Fig. 5-2).
**Fig. 5-2.** The Tolloid autoregulatory loop in DV patterning in equilibrium. A see-saw model depicting the pathway that self-regulates Tolloid activity in the developing gastrula. Black arrows represent extracellular protein-protein interactions, while blue arrows represent transcriptional regulation. BMP ligands (BMPs) activate the transcription of Sizzled in the ventral Center. Crescent and Chordin are secreted from the dorsal center in areas of low BMP signaling. Chordin binds BMPs in the extracellular space, preventing the interaction of BMP with its receptors, thus inhibiting BMP signaling. Tolloid metalloproteinases cleave Chordin, which increases free BMPs levels, resulting in increased BMP signaling. Crescent and Sizzled inhibit Tolloid metalloproteinases, leading to increased Chordin levels and BMP signaling. In conclusion, the levels of Chordin, the key regulator of BMP signaling in the developing gastrula, is maintained by Crescent and Sizzled from the dorsal and ventral pole respectively, through the inhibition of the proteolytic activity of Tolloid metalloproteinases.
The Crescent/Sizzled Pair Self-Regulates upon Perturbations of the System

When Crescent is overexpressed, for example by microinjection of Crescent mRNA, the reduction in Tolloid activity results in increased Chordin levels and lower BMP signaling (Fig. 5-3, panels A and B). This in turn will lead to a decrease in transcription of Sizzled on the ventral pole. This response allows Tolloid to regain activity and the system to equilibrate, compensating the increase in Crescent levels by a decrease in Sizzled production. Similarly, when Crescent is underexpressed, which is the case in embryos challenged with Crescent MO injections, Chordin levels will fall due to an increased activity of Tolloid metalloproteinases, and free BMPs will result in stronger and ectopic BMP signaling. This will, in turn, increase the transcription of Sizzled, which will attenuate the BMP signals by protecting Chordin from Tolloid-driven proteolytic degradation (5-C, panel C). Thus, the system is wired in a way that perturbations in Crescent levels are compensated by an increase or decrease in Sizzled transcription (Ploper et al., 2011).

Similarly, upon perturbations of Sizzled levels the dorsal ventral signaling network is also equipped with mechanisms to self-regulate. When Sizzled is overexpressed in embryos microinjected with Sizzled mRNA, or knocked-down in embryos challenged with Sizzled MO, changes in BMP signaling trigger a negative feed-back mechanism which activate or repress Sizzled transcription, directly compensating for the fluctuations in Sizzled levels (Fig. 5-4). In the specific case of Sizzled MO, the transcriptional response to this perturbation is unsuccessful since the morpholino will block further Sizzled translation. This explains the high penetrance and strong ventralized phenotypes observed in Sizzled MO-injected compared to Crescent MO-
Fig. 5-3. Sizzled levels compensate for perturbations in Crescent. Full lines indicate increased activity, while dotted lines indicate diminished activity, of a given interaction. The initial perturbation to the system is indicated in red, while the response of the system is indicated in green.

(A) In a state of equilibrium, the DV patterning network creates and maintains the BMP signaling gradient. An in situ hybridization shows Sizzled transcript levels in uninjected control embryos.

(B) Upon Crescent mRNA injections, Tolloid activity is inhibited and Chordin protein levels increase. This in turn leads decreased BMP signaling and less Sizzled transcription. Ultimately, the inhibition of Sizzled transcription will equilibrate the system by causing an increase in Tolloid activity. In this way, the system responds to an increase in Crescent levels by decreasing Sizzled transcription, which can be observed in whole mount in situ hybridization on Crescent mRNA-injected embryos.

(C) If Crescent is depleted, as in the case of Crescent MO injected embryos, the increase in Tolloid activity and consequent increase in cleavage of Chordin lead to increased BMP activity. This, in turn, activates more Sizzled transcription which will equilibrate the system by inhibiting Tolloids, protecting Chordin from proteolytic digestion. Embryos challenged with Crescent MO show a massive increase in Sizzled transcripts, as observed by whole mount in situ hybridization for Sizzled transcripts, in an effort to compensate for Crescent and decrease BMP signaling.
injected embryos. This also illustrates the importance of Sizzled in DV patterning robustness (Yabe et al., 2003; Lee et al., 2006).

One classical embryological manipulation that illustrates the robustness of DV patterning is the physical bisection of early *Xenopus* gastrula embryos into dorsal and ventral halves. Amazingly, the dorsal halves, which contain the Spemann organizer, are capable of re-patterning their tissues and develop into a well-proportioned organisms, albeit scaled to a smaller size (Fig 2-2 panel A), while the ventral halves are destined to remain a mass of ventral tissues, called “belly piece” or “Bauchstück”. This ability of the dorsal piece to dynamically adjust patterning to accommodate its new reduced size is termed scaling (Ben-Zvi et al., 2011). Recently, Sizzled was shown to be very important for this regeneration of pattern (Inomata et al., 2013). Sizzled levels, were found to be coupled to embryo size, and to dynamically control proteolytic degradation of Chordin in order to proportionally adjust the BMP gradient to embryonic axis size (Inomata et al., 2013). Upon removal of ventral tissues during dorsal-ventral bisections, the elimination of ventral tissues and reduction of Sizzled generates a steeper gradient of Chordin. Although the role of Crescent in scaling remains unexplored, the Crescent/Sizzled sFRP pair constitute an essential component of the extracellular circuit of secreted proteins that read aberrant BMP signaling in the developing gastrula and, through transcriptional feed-back loops, adjust the degradation of Chordin, a process which is at the heart of DV patterning.
Fig. 5-4. The resilience of the DV network: Crescent and Sizzled.

(A and B) Crescent levels and Tolloid activity are corrected by BMP-driven Sizzled transcription.

(C) In Sizzled mRNA-injected embryos, Tolloid activity is greatly reduced, and Chordin protein levels accumulate. This prevents BMP signaling, and hinders further Sizzled transcription. In this manner, the transcription of Sizzled is regulated by a negative feed-back loop.

(D) When Sizzled is knocked down using a Sizzled MO, the negative feed-back loop involving Toloids/Chordin/BMP will lead to higher BMP signaling, in an attempt to restore Sizzled levels and lower BMP signaling. However, in Sizzled MO-injected embryos this response is abrogated by the morpholino, resulting in extremely ventralized phenotypes.
The MITF Family of Transcription Factors in Lysosomal Biogenesis and Disease

The conclusions and perspectives of the work on MITF, lysosomal biogenesis, and Wnt signaling are presented here in the form of a scholarly review that I wrote by invitation of the editor of Pharmacological Research. It is concerned with the effects of the MiT family of transcription factors in oncogenesis, focusing on lysosomal biogenesis, canonical Wnt signaling, and the stabilization of Glycogen Synthase Kinase (GSK3) substrates.

MITF, a melanoma oncogene member of the Microphthalmia family of transcription factors (MiT), together with the other MiT members TFEB and TFE3, were known to be amplified and overexpressed in diverse cancers and behave as oncogenes. Only recently, their physiological role as regulators of cellular clearance pathways has been revealed. In the case of MITF, its function in lysosomal biogenesis and its phosphorylations at Wnt/GSK3 regulated sites were unknown until our work (Ploper et al., 2015). MiT proteins achieve this by transcriptionally regulating the biogenesis of lysosomes and autophagosomes through activation of Coordinated Lysosomal Expression And Regulation (CLEAR) elements in promoters of target genes. Soon after the role MITF in lysosomal biogenesis was published (Ploper et al., 2015), ChIP-seq data independently confirmed that endogenous MITF, together with the chromatin remodeller BRG1, occupied promoters of lysosomal genes in melanoma cells (Laurette et al., 2015).

The underlying oncogenic mechanism of the MiT family has remained elusive. I have reviewed the possible mechanisms involved in their role as oncogenes taking into account their newly discovered roles in lysosomal biogenesis and rewiring of the lysosomal pathway. In melanomas, there is evidence that MITF amplification could lead to an inhibition of autophagy, bypassing cellular senescence response mechanisms, and an enhancement of Wnt signaling through increased sequestration of Wnt signaling components in multivesicular bodies (MVBs).
What follows is the text and figures of a review, of which I am the corresponding author (Ploper and De Robertis, 2015):

The MITF family of transcription factors: role in endolysosomal biogenesis, Wnt signaling, and oncogenesis


Diego Ploper* and Edward M. De Robertis

ABSTRACT

Canonical Wnt signaling influences cellular fate and proliferation through inhibition of Glycogen Synthase Kinase (GSK3) and the subsequent stabilization of its many substrates, most notably β-Catenin, a transcriptional co-activator. MITF, a melanoma oncogene member of the Microphthalmia family of transcription factors (MiT), was recently found to contain novel GSK3 phosphorylation sites and to be stabilized by Wnt. Other MiT members, TFEB and TFE3, are known to play important roles in cellular clearance pathways by transcriptionally regulating the biogenesis of lysosomes and autophagosomes via activation of CLEAR elements in gene promoters of target genes. Recent studies suggest that MITF can also upregulate many lysosomal genes. MiT family members are dysregulated in cancer and are considered oncogenes, but the underlying oncogenic mechanisms remain unclear. Here we review the role of MiT members, including MITF, in lysosomal biogenesis, and how cancers overexpressing MITF, TFEB or TFE3 could rewire the lysosomal pathway, inhibit cellular senescence, and activate Wnt signaling by increasing sequestration of negative regulators of Wnt signaling in multivesicular bodies (MVBs). Microarray studies suggest that MITF expression inhibits macroautophagy. In melanoma the MITF-driven increase in MVBs generates a positive feedback loop between MITF, Wnt, and MVBs.
1. Introduction - Wnt Signaling

The Wnt signaling pathway plays fundamental roles in cell biology. During development, Wnt regulates stem cell differentiation, embryonic patterning and organogenesis (Clevers and Nusse, 2012; Hikasa and Sokol, 2013; Holland et al., 2013). Wnt also regulates adult tissue homeostasis, and dysregulation of this pathway often leads to diseases such as cancer, in which Wnt signaling is frequently hyperactivated (Clevers and Nusse, 2012; Anastas and Moon, 2013). In the absence of Wnt signaling, β-Catenin is phosphorylated by Casein Kinase 1 α (CK1 α) and Glycogen Synthase Kinase 3 (GSK3) in a “destruction complex” which also contains the scaffold proteins Adenomatous Polyposis Coli (APC) and Axin1 (Clevers and Nusse, 2012). These successive phosphorylations are recognized as a phosphodegron by Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase (β-TRCP), which ubiquitinates β-Catenin and targets it for proteasomal degradation. In this way, without Wnt stimulation, the destruction complex maintains cytosolic β-Catenin levels in check. When cells are subject to Wnt
stimulation, Wnt ligands bind to their receptors low-density lipoprotein receptor-related 5 or 6 (LRP5/6) and Frizzled (Fz), resulting in the recruitment of the destruction complex, clustering, and subsequent endocytosis of activated receptor complexes into “Wnt-signalosomes” (Bilic et al., 2007).

This complex is then trafficked inside multivesicular bodies (MVBs) in an Endosomal Sorting Required for Transport (ESCRT)-dependent manner (Taelman et al., 2010; Vinyoles et al., 2014; Ploper et al., 2015). The sequestration of GSK3 and Axin inside MVBs is required for Wnt signaling, highlighting the importance of late endosomes as positive regulators of growth factor signalling (Taelman et al., 2010; Dobrowolski and De Robertis, 2011). In this way, the destruction complex is sequestered from the cytosol and newly-synthesized β-Catenin is allowed to accumulate and translocate to the nucleus, where it acts as a transcriptional co-activator that regulates the transcription of Wnt-target genes.

2. A plethora of GSK3 phosphorylations

Wnt signaling promotes stabilization of many proteins in addition to β-Catenin (Kim et al., 2009; Taelman et al., 2010; Acebron et al., 2014). A bioinformatic screen of the human proteome revealed that up to 20% of human proteins contain three or more putative consecutive GSK3 phosphorylation sites (much more than predicted by chance alone), which have the potential of bringing the degradation of many cellular proteins under the control of Wnt (http://www.hhmi.ucla.edu/derobertis/EDR_MS/GSK3%20Proteome/Table_1-full_table.xls) (Taelman et al., 2010). As cells need to conserve their proteins in preparation for cell division, Wnt signaling is particularly strong at the G2/M of the cell cycle (Davidson et al., 2009). Furthermore, Wnt signaling can promote cell size growth independently of β-Catenin in cancer
cells with constitutively stabilized β-Catenin. This new branch of canonical Wnt signaling, independent of β-Catenin-driven transcriptional effects, has been recently designated the Wnt-dependent STabilization Of Proteins (Wnt/STOP) pathway (Acebron et al., 2014).

Analysis of the putative GSK3-regulated phosphoproteome revealed that the microphthalmia-related (MiT) family of basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factors possessed the most phylogenetically conserved GSK3 phosphorylation sites (Ploper et al., 2015). As shown in Fig. 5-5, three consecutive phosphorylation sites are present in the highly conserved carboxy-terminus of the MiT member MIcrophthalmia-associated Transcription Factor (MITF) as well as in its paralogues TFEB, TFE3, and TFEC. MITF is the master regulator of melanocytes, and its levels act as a rheostat to control cellular proliferation in melanomas (Setingrimsson et al., 2004; Carreira et al., 2006; Strub et al., 2011). Using a custom-made phospho-specific antibody, these residues were shown to be phosphorylated by GSK3 and to mediate Wnt-induced protein stability, underscoring the predictive capacity of the putative GSK3 phosphoproteome, and reinforcing the concept of the Wnt/STOP pathway (Ploper et al., 2015).
MITF contains three novel GSK3 phosphorylation sites in its C-terminal region (in red), which can be primed by p90/RSK1 phosphorylation (in blue). The p90/RSK enzyme is also known as MAPK-Activated Protein Kinase 1, or MAPKAP-K1, and is a downstream target of the Ras/Erk signaling pathway. The GSK3 sites, as well as the priming phosphorylation site are present in the other members of the MiT family (TFEB, TFE3, TFEC). These GSK3 sites have the highest degree of conservation for GSK3 sites in phylogenetic proteome analyses, and are even found in insects (Ploper et al., 2015).
3. The MiT Family of transcription factors

The Microphthalmia family of bHLH-LZ transcription factors (MiT) is composed of four members: MITF, TFEB, TFE3 and TFEC (Steingrímssson et al., 2004; Arnheiter et al., 2007). These proteins can form homo- or hetero-dimers through their HLH-LZ motif with members within the family, but not with other related bHLH proteins such as c-Myc, Max or USF (Hemesath et al., 2004; Pogenberg et al., 2012). Their basic domain enables them to bind to DNA, specifically E-boxes (Ephrussi boxes) and regulate the transcription of target genes involved in a wide range of different cellular processes, from pluripotency (Betschinger et al., 2013) and cell specific differentiation (Steingrímssson et al., 2004) to basic cellular homeostasis (Settembre and Ballabio, 2014). Importantly, MiT family members are frequently dysregulated in many forms of cancer (Haq and Fisher, 2011).

3.1. MITF

MITF is an evolutionarily conserved transcription factor with homologs in Caenorhabditis elegans and Drosophila (Hallsson et al., 2004). Paula Hertwig described the first MITF mutation 70 years ago, when she found that progeny of X-ray irradiated mice had white coat color and small eyes (Hertwig, 1942). Since then, much has been discovered about MITF, resulting in a vast body of literature (Steingrímssson et al., 2004; Arnheiter et al., 2007).

The mitf gene is predominantly expressed in melanocytes and retinal pigment epithelial cells (RPE), but is also expressed in a variety of other cell types (osteoclasts, natural killer cells, macrophages, mast cells, B cells, and cardiac muscle cells) (Arnheiter et al., 2007). MITF is subject to differential splicing and differential promoter usage, giving rise to multiple isoforms.
that differ in their first exon. Many of these isoforms show a tissue-restricted expression pattern. For example, MITF-M is an isoform preferentially expressed in melanoblasts and melanocytes, MITF-D is mainly expressed in the RPE and monocyte lineage (Takeda et al., 2002), and MITF-mc is expressed in mast cells (Takemoto et al., 2002), while MITF-A and MITF-H are more ubiquitous, being expressed in several tissues (Steingrimsson et al., 2004; Arnheiter et al., 2007).

Most of the current knowledge on MITF comes from studies in melanocytes and melanomas, in which the MITF-M isoform functions as a master gene regulator of the melanocytic lineage required for the development, growth and survival of melanocytes (Steingrimsson et al., 2004; Arnheiter et al., 2007). Mutations in the mitf gene can give rise to mice that have a white coat color, are deaf, and present microphthalmia (Steingrimsson et al., 2004; Arnheiter et al., 2007). In humans, heterozygous mutations in mitf can cause Waardenburg Syndrome type 2, a hair pigmentation and deafness disorder (Read et al., 1997), and dominant-negative mutations cause Tietz syndrome, a closely related condition characterized by generalized depigmentation and deafness (Smith et al., 2000). Importantly, genomic amplification of MITF is observed in 15-20% of melanomas, in which it functions as a lineage addiction oncogene (Garraway et al., 2005).

3.2. TFEB

Transcription Factor E Box (TFEB), the second best characterized member of the MiT family, plays a crucial role in cellular homeostasis and has been recently described as the master regulator of cellular clearance pathways (Palmieri et al., 2011; Settembre et al., 2013). TFEB achieves this by transcriptionally upregulating genes involved in lysosome and autophagosome biogenesis, as well as by activating lysosomal exocytosis and lipid metabolism (Sadiello et al.,
TFEB provides a link between the nutrient-sensing mechanistic Target of Rapamycin Complex 1 (mTORC1) machinery and the transcriptional cellular response needed to cope with nutritional stress. In nutrient-rich conditions, active mTORC on the outer lysosomal membrane phosphorylates TFEB, inhibiting its nuclear translocation. Upon starvation, mTORC is inhibited and unphosphorylated TFEB accumulates in the nucleus, where it binds to Coordinated Lysosomal Expression And Regulation (CLEAR) sequences located in the promoters of lysosomal genes and activates the expression of the suite of genes necessary for cells to survive nutrient deprivation (Settembre et al., 2013; Sardiello and Ballabio, 2009).

3.3. TFE3

Like TFEB, Transcription Factor E3 (TFE3) has been shown to participate in the biogenesis of lysosomes and autophagosomes and the clearance of cellular debris under conditions of starvation or lysosomal stress (Martina et al., 2014). The activity of TFE3 is also dictated by its nuclear localization, which is regulated by mTOR and nutrient levels. When active, mTOR phosphorylates TFE3 causing its cytoplasmic retention. In conditions of nutritional deprivation, mTORC is inhibited and unphosphorylated TFE3 can enter the nucleus and activate the transcription of genes containing CLEAR elements in their promoters (Martina et al., 2014). TFE3 has also been recently described to play a role in the maintenance of pluripotency (Betschinger et al., 2013). A relocalization of TFE3 from the nucleus to the cytoplasm was found to be required for embryonic stem cells (ESC) to exit their naïve pluripotent state and commit to cellular differentiation. The cytoplasmic retention of TFE3 is controlled by the activity of the tumor suppressor Folliculin, its binding partners Folliculin-
interacting proteins 1 and 2 (Fnip1/2), and the mTORC regulator TSC2 (Hong et al., 2010; Betschinger et al., 2013).

3.4. TFEC

Of the four members of the MiT family, TFEC is the least studied one. Its expression is restricted to macrophages (Rehli et al., 1999), and its function has not been widely investigated. Furthermore, while mouse and rat TFEC are devoid of the activation domain required for promoting transcription (Zhao et al., 1993; Rehli et al., 1999a; Rehli et al., 1999b), the human homologue does contain this activation domain and is able to activate expression of a reporter gene construct driven by the tyrosinase or heme oxygenase-1 gene promoters (Yasumoto et al., 1997).

4. MiT transcription factors drive lysosome and autophagosome biogenesis

As mentioned above, TFEB and TFE3 have been clearly established as regulators of lysosomal biogenesis. TFEB and TFE3 are recruited to the outer lysosomal membrane by interacting, through their 30-most N-terminal residues, to Rag guanosine triphosphatases (GTPases) (Martina and Puertollano, 2013). In nutrient-rich conditions, active mTORC1 is recruited to the surface of lysosomes (Efeyan et al., 2015), where it phosphorylates Ser142 in TFEB (and also possibly Ser211 in TFEB and Ser321 in TFE3), triggering the binding of these transcription factors to 14-3-3 cytosolic chaperones (Settembre et al., 2012). This causes cytoplasmic retention of TFEB and TFE3, resulting in decreased transcriptional activity (Settembre et al., 2013; Martina et al., 2014). However, when cells are subject to nutritional challenges (such as amino acid starvation) or lysosomal stress (such as chloroquine treatment),
mTORC activity is inhibited, and unphosphorylated TFEB and TFE3 translocate to the nucleus, bind to CLEAR elements, and drive the coordinated transcription of gene programs that restore nutritional homeostasis through an increase in lysosomes, autophagosomes, and exocytosis (Settembre et al., 2013; Martina et al., 2014).

**5. MITF expression correlates with many endolysosomal genes**

In recent work, MITF has also been implicated in lysosomal biogenesis (Ploper et al., 2015). Using Gene Set Enrichment Analysis (GSEA) of microarray expression datasets from 51 different melanoma lines analyzed at UCLA (Ploper et al., 2015), MITF levels were found to correlate with the expression of many, but not all lysosomal genes containing CLEAR elements in their promoters/enhancers (Fig. 5-6). Intriguingly, TFEB levels did not correlate with lysosomal gene expression in melanoma microarray datasets (p = 0.276) (Ploper et al., 2015). Furthermore, overexpression of MITF-M was able to drive transcription of lysosomal markers and activate a CLEAR element reporter in melanoma cells. Surprisingly, despite a robust increase in endolysosomes/MVBs induced by MITF-M, these organelles are not completely functional lysosomes because they failed to degrade endocytosed proteins (Ploper et al., 2015).

There were other indications suggesting a role for MITF isoforms in regulating CLEAR element lysosomal genes. MITF-A has been shown to localize at the lysosomal membrane, bind to Rag GTPases, and translocate to the nucleus upon mTOR inhibition, mimicking the nutrient-driven regulation of TFEB and TFE3 (Martina and Puertollano, 2013). In the case of TFEB, Ser142 and Ser211 were known to be required for cytoplasmic retention, as they are the sites for mTORC phosphorylations which promote association with 14-3-3 proteins (Settembre et al., 2012; Rocznik-Ferguson et al., 2012). Interestingly, Ser173 in MITF, which is analogous to
Fig. 5-6. In melanoma cell lines MITF expression correlates with the expression of many lysosomal genes containing a CLEAR promoter element. This microarray analysis shows that 51 melanoma cell lines clustered into two distinct groups according to their expression signature of 63 CLEAR element lysosomal genes (Sardiello et al., 2009). One group had high MITF expression (in red) and the other had low MITF expression (in green). The melanoma group with high MITF expression levels upregulated many, but not all, lysosomal genes containing a CLEAR element in their promoter. Horizontal lines in this heat map correspond to individual cell lines, and vertical lines show lysosomal gene expression levels. MITF expression was added as a reference, for it is not a lysosomal gene. The cell lines were allowed to sort according to their lysosomal gene expression signature. Surprisingly, all cell lines with high MITF mRNA expression (mostly through MITF amplification) clustered as a high lysosomal gene expressing group (above the dotted white line). Reproduced from the supplementary information of Ploper et al., 2015.
Ser211 in TFEB, has also been shown to be required for cytoplasmic retention by interacting with 14-3-3 proteins in myeloid precursors (Bronisz et al., 2006). It is important to note that while the activity of TFEB, TFE3, and MITF isoforms A and D is regulated by nutritional status which determines cytoplasmic versus nuclear localization through mTOR phosphorylations, the MITF-M isoform, which is the one enriched in melanomas, escapes this regulation as it lacks the amino-terminal domain required for binding to Rag GTPase and lysosomal docking (Rocznia-Ferguson et al., 2012; Martina and Puertollano, 2013). Thus, MITF-M is a constitutively nuclear transcription factor. MITF-A overexpressed in the human retinal pigment epithelium cell line ARPE-19 effectively activates the expression of many autophagy genes in this cell type (Martina et al., 2014).

6. MITF expression negatively correlates with key macroautophagy genes

Autophagosomal genes did not significantly correlate with MITF expression. In Supplementary Table 1 we list the rank order of 13,214 genes arranged according to their degree of correlation with MITF across 83 different melanoma cell lines in microarrays. Examination of 39 autophagosomal genes (Settembre et al., 2011) showed that while some were positively correlated (ATG3, UVRAG, VPS11, SNCA, and AMBRA1) many others (e.g., ATG7, ATG12, ATG5) were very negatively correlated with MITF. In GSEA analyses (Subramanian et al., 2005) the set of autophagy genes had no significant correlation (p=0.314) with MITF, while lysosomal genes were highly correlated (p < 0.001) (Ploper et al., 2015).

The observation that the MITF in melanomas (MITF-M) correlates with lysosomal genes but with not autophagy genes is surprising, given the fact that the CLEAR element (-CTCACGTGAG-) contains one of the two DNA binding sequences for MITF (-CACGTG-), as...
defined from ChIP-seq experiments from melanoma cells (Sardiello et al., 2009; Strub et al., 2011). In addition, the CLEAR element also contains a 5’ flanking T known to be crucial for MITF binding (Aksan and Goding, 1998). A further layer of mystery is added by the fact that in RPE cells MITF-A, which can be regulated by nutrient starvation, activates autophagy but not lysosomal genes (Martina et al., 2014).

ChIP-seq experiments in melanoma have uncovered potential roles for MITF in regulating DNA replication, mitosis, and genomic stability. In these datasets MITF was found bound to promoters of many lysosomal genes (Strub et al., 2011). Furthermore, DNA microarray analysis of MITF targets had also identified lysosomal genes such as acid Ceramidase (ASAH1), GM2 ganglioside activator (GM2A), and various ATPases (Hoek et al., 2008). Finally, MITF was known to activate transcription of the lysosomal genes acid phosphatase 5 (ACP5), chloride channel voltage-sensitive 7 (CLCN7), osteopetrosis-associated transmembrane protein 1 (OSTM1) and cathepsin K (Luchin et al., 2000; Motyckova et al., 2001; Meadows et al., 2007). Although MITF is known to regulate the expression of many genes required for melanosome biogenesis, and melanosomes are frequently referred to as a lysosome-related organelle (LRO), melanosomes are quite different from conventional endosomes and lysosomes, and represent a distinct lineage of organelles with a unique proteomic profile (Raposo et al., 2001; Chi et al., 2006; Raposo and Marks, 2007; Lübke et al., 2009). Thus, the transcriptional regulation of the lysosomal gene set by MITF is not merely an upregulation of melanosomal genes.

Since MiT members can bind to similar core DNA sequences and hetero-dimerize with each other, there are possibilities for crosstalk and compensation among members. For example, MITF and TFE3 have been shown to be redundant in osteoclasts (Steingrímsson et al., 2002). In conclusion, the MiT family members TFEB, TFE3 and MITF regulate lysosomal and autophagy
genes, but the contribution of each member, or each isoform in the case of MITF, to organelle biogenesis may depend on nutritional conditions, cell type and signaling inputs.

7. The MiT family in cancer

MITF, TFEB and TFE3 are oncogenes. MITF is amplified in 20% of melanomas, while TFEB and TFE3 are driven by translocations in pediatric renal cell carcinomas and alveolar soft part sarcomas (Haq and Fisher, 2011). In pediatric renal carcinomas, TFE3 is translocated in up to 30-50% of cases (Gunawan et al., 2003; Ramphal et al., 2006).

Through systematic analysis of cancer genomes, melanomas were found to have amplifications of chromosome 3p14-3p12, which coincides with the MITF locus (Garraway et al., 2005). MITF is amplified in 20% of metastatic melanomas, one of the most aggressive and chemo resistant cancers known, and associated with decreased patient survival. Furthermore, overexpression of MITF is able to cooperate with BRAFV600E activating mutations in transforming human melanocytes in vitro. This places MITF in the category of a “lineage survival”, or “lineage addiction” oncogene, since in addition to its oncogenic effects it is required for the development and survival of the melanocytic lineage (Garraway et al., 2005; Garraway and Lander, 2013).

A point mutation in MITF, E318K, predisposes to familial and sporadic melanoma (Yokoyama et al., 2011). This mutant MITF protein cannot be properly SUMOylated and has increased transcriptional activity (Yokoyama et al., 2011; Bertolotto et al., 2011). The ability of MITF to promote oncogenesis is not restricted to the melanocyte lineage, since the MITF$^{E318K}$ mutant also predisposes to renal cell carcinoma (Bertolotto et al., 2011). Furthermore, MITF is ectopically overexpressed in clear cell sarcomas when its transcription is activated by a fusion
protein resulting from chromosomal translocation of the Ewing Sarcoma protein (EWS) to Activating Transcription Factor 1 (ATF1) (Zucman et al., 1993). Although these sarcomas do not have MITF genomic rearrangements, they are entirely dependent on MITF transcription and activity for proliferation (Davis et al., 2006).

In melanomas, MITF is known to act as a molecular rheostat; at very low levels it will causes cellular senescence, while at very high levels it can drive differentiation and growth arrest. At intermediate/high levels, however, MITF promotes cellular growth (Steingrímsson et al., 2004; Carreira et al., 2006; Strub et al., 2011). MITF has been proposed to promote oncogenesis by influencing multiple cellular processes, regulating the expression of genes involved in proliferation, survival, motility, oxidative stress and DNA repair, all of which are key processes in carcinogenesis (Haq and Fisher, 2011).

MITF-M overexpression in melanoma cells causes an expansion of the late endolysosome/MVB compartment which enhances Wnt signaling by increasing the sequestration of destruction complex components, including Axin1, GSK3, and phospho-β-Catenin inside MVBs (Ploper et al., 2015). As depicted in Fig. 5-7, in the presence of Wnt a positive feedback loop is generated, in which MITF stabilized by Wnt increases MVB biosynthesis which in turn potentiates Wnt signaling (Ploper et al., 2015).

A recent study found that melanomas rely on lysosomal function more than other cancers (Alonso-Curbelo et al., 2014). Interestingly, the related MiT transcription factors TFEB and TFE3, master lysosomal regulators, can rescue MITF knockdown in MITF-driven clear cell sarcomas (Davis et al., 2006). Likewise, knockdown of TFE3 inhibits growth in TFE3-translocated renal cell carcinomas while co-transfection of ectopic MITF restores proliferation in these cells (Davis et al., 2006). In renal cell carcinomas, translocated TFEB transcription is
Fig. 5-7. Diagram showing how MVB/endolysosomal membrane trafficking regulates MITF and other Wnt/STOP targets through the sequestration of GSK3 and Axin. Upon Wnt ligand binding, the destruction complex that phosphorylates and targets β-Catenin for proteasomal degradation is recruited to the LRP6 receptor complex, which is endocytosed and subsequently sequestered inside intraluminal vesicles of multivesicular bodies (MVBs). The sequestration of GSK3 and Axin1 stabilizes MITF protein, which can constitutively translocate into the nucleus and promote expression of a large subset of lysosomal genes. This expands the MVB compartment which can further sequester destruction complex components and enhance the Wnt signal. In the absence of Wnt, GSK3 phosphorylates MITF at the C-terminal sites, promoting its degradation in proteasomes.
driven at high levels and is required for the growth of these tumors ((Davis et al., 2006; Kuiper et al., 2003). Recently, pancreatic cancer cell lines were shown to have nuclear localization of TFEB despite culture in a nutrient-rich medium while non-transformed pancreatic epithelial cells had cytoplasmic TFEB, suggesting a role of this lysosome-inducing transcription factor in promoting pancreatic cancer cell growth (Marchand et al., 2015).

Taken together, these studies argue that MiT family members are oncogenes that can compensate for each other in oncogenesis. It is unclear whether MITF, TFEB, and TFE3 are promoting oncogenic growth by targeting a common pathway or gene network in different tumors. However, it seems likely that a reprogramming of the endolysosomal/autophagic pathway is taking place given the recent findings linking MITF and other MiT members in lysosome/autophagosome biogenesis. The nature and mechanistic detail of this rewiring, and how it affects cellular proliferation and metabolism merits further investigation.

8. Lysosomes and autophagosomes in melanomas

Cancer cells have alterations in lysosomal/autophagosome composition and abundance that may participate in oncogenesis by promoting invasive growth, metabolism and angiogenesis (Kallunki et al., 2012). Among all cancers, melanomas rely on the endolysosomal pathway for growth more strongly than others, and lysosomal genes (plus lysosomal-associated genes such as Rab7) are more highly expressed in melanomas compared to other tumors (Alonso-Curbelo et al., 2014). Rab7, a small GTPase which has pivotal roles in lysosomal biogenesis and the degradation of lysosomal-associated vesicles (Bucci et al., 2000; Zerial et al., 2001; Zhang et al., 2009), has been recently shown to be an early melanoma driver capable of rewiring the endolysosomal pathway in a lineage-specific fashion (Alonso-Curbelo et al., 2014). Although in
this study Rab7 levels were reported to be independent of MITF, MITF significantly correlates with Rab7 in microarray data presented in Supplementary Table 1 (in which it appears at position 226/13,214; p < 0.001), as well as with many lysosomal genes (Ploper et al., 2015). It appears that Rab7- or MITF-driven reprogramming of the endolysosomal pathway may confer growth advantages to melanomas. It is likely that other tumors that overexpress TFEB and TFE3 due to chromosomal translocations may also rewire the endolysosomal pathway.

Macroautophagy is a vital cellular process by which cells digest their own contents by engulfing cellular materials in double-membrane vesicles, called autophagosomes that then fuse to lysosomes (Mizushima and Komatsu, 2011). The role of macroautophagy in cancer is complex (Kubisch et al., 2013; Galluzzi et al., 2015). Although it is generally considered a tumor-suppressing mechanism, it can also replenish nutrients required by rapidly growing tumor cells enabling further growth (White, 2012; White, 2015). Autophagy is regulated by highly conserved autophagy-related proteins (ATGs) required for initiation of autophagosome formation, cargo collection and trafficking to the lysosomal compartment (Mizushima, 2007). Autophagy-Related Protein 5 (ATG5), an E3 ubiquitin ligase required for autophagosome elongation, has recently been shown to be downregulated in melanomas compared to nevi and melanocytes (Liu et al., 2013). This is accompanied by a general inhibition of autophagy. When ATG5 is ectopically expressed in melanoma cells, it is capable of inhibiting proliferation and inducing senescence, as well as increasing autophagy (Liu et al., 2013). In this way, autophagy in general and ATG5 in particular function as tumor suppressors in melanoma (Liu et al., 2013). Interestingly, high MITF expression levels correlate with very low ATG5 and ATG7 levels in microarray expression data from a panel of 83 melanoma cell lines (appearing at positions 11,730 and 12676, respectively, in Supplementary Table 1, p < 0.001). Macroautophagy
contributes to the induction of oncogene-induced cell senescence, and melanomas with impaired autophagy and reduced ATG5 levels may escape this homeostatic mechanism (Liu et al., 2013; Liu et al., 2014a; Liu et al., 2014b). High levels of MITF, which correlate with low levels of ATG5, might contribute to proliferation by inhibiting oncogene-induced senescence (Giuliano et al., 2010; Giuliano et al., 2011). In conclusion, MITF expression drives lysosomal biogenesis but represses key macroautophagy genes in melanoma (Table 1).

MITF, although capable of inducing late endosome/MVB formation, does not induce proteolytically active lysosomes (Ploper et al., 2015). The MITF-induced increase in MVBs in turn enhances Wnt signaling by increasing the sequestration of destruction complex components such as GSK3 and Axin, which is relevant in melanoma (Ploper et al., 2015; Biechele et al., 2012), resulting in increased MITF stabilization (Fig. 5-8). It is possible that cancers in which TFEB and TFE3 are overexpressed also expand the late endolysosomal compartment causing an increase in Wnt signaling by a similar mechanism. Chloroquine (CQ), a lysosomotropic weak base known to inhibit lysosomal acidification and function (Rote and Rechsteiner, 1983) also enhances Wnt signaling by increasing GSK3 sequestration (Dobrowolski et al., 2012).

9. Pharmacological approaches

Lysosomal and autophagosomal function is critical for the maintenance of proteostasis and for the ability of cells to respond to stress (White, 2015; Liu et al., 2014a; Liu et al., 2014b; Manic et al., 2014). Although autophagy has been described as a tumor suppressing mechanism (given that loss of ATG genes promote cancer, including lymphomas and lung and liver carcinomas (Liang et al., 1999; Qu et al., 2003; Yue et al., 2003), CQ and hydroxychloroquine (HCQ) (FDA-approved drugs for the prophylactic treatment of malaria, lupus erythematosus and rheumatoid arthritis) have never been linked to malignant transformation. Autophagy is also
Fig. 5-8. Model of the positive feed-back loop through which MITF increases Wnt responsiveness by the expansion of the MVB compartment. Expansion of the MVB compartment causes increased inhibition of GSK3 by enhancing its sequestration inside the late endosomal compartment. This in turn inhibits the C-terminal phosphorylations of MITF, increasing its stability and activity.
crucial for supporting progression and dissemination of established tumors, allowing cells to survive in low-nutrient and hypoxic conditions (Cuervo, 2004). Therefore, the lysosomal/autophagosomal pathway has long been considered an attractive target for anticancer treatments. Inhibitors of lysosome/autophagosome function such as CQ, HCQ, Lys0569, Monensin, Bafilomycin A1, or of PI3K (Wortmannin) have been proposed to have possible antineoplastic effects, particularly in combination with conventional chemotherapy treatments (Manic et al., 2014; Corazzari et al., 2013).

Although most melanomas are initiated by activating BRAF V600E mutations (also present in benign nevi), MITF expression is a key driver of melanoma proliferation (Wellbrock and Arozarena, 2015). At intermediate levels in the MITF rheostat, melanoma cells actively proliferate, have elevated Wnt signaling, and tend not to metastasize (Hoek et al., 2008). However, at low MITF levels melanoma cell phenotypes switch to a slow-proliferating stem cell-like cells with metastasizing properties. Melanomas progress through cell switching between these two cellular phenotypes that coexist in tumors in vivo (Hoek et al., 2008). Recently, MITF-driven phenotype switching has been proposed as of possible therapeutic value. Induction of MITF causes expression of high levels of the enzyme Tyrosinase, the rate limiting enzyme in the synthesis of melanin. An irreversible inhibitor of dihydrofolate reductase (DHFR) has been engineered as a drug precursor (called TMECG) that is active only after being processed specifically by Tyrosinase (Sáez-Ayala et al., 2013). Post-translational Wnt/STOP signaling, through GSK3 inhibition, stabilizes MITF (Ploper et al., 2015), and MITF is also transcriptionally upregulated by β-Catenin (Takeda et al., 2000). This suggests the possibility that it might be possible to combine the use of GSK3 inhibitors (which increase MITF activity
and consequently the expression of its target gene Tyrosinase), with the DHFR inhibitor TMECG in order to boost the sensitivity of the chemo-resistant invasive stem-like cells to treatment.

In addition, GSK3 inhibitors might enhance the effects of CQ on tumors highly dependent on endolysosomal activity, such as is the case in melanomas (Alonso-Curbelo et al., 2014). Inhibiting GSK3 activity with small molecules may be considered a double-edged sword as it could stimulate malignant transformation and cancer growth by activating Wnt/β-Catenin signaling or by stabilizing oncogenes regulated by Wnt/STOP such as c-Jun and c-Myc (Taelman et al., 2010; Acebron et al., 2014). However, long-term studies in mice treated with GSK3 inhibitors have not revealed oncogenic effects (McCurbey et al., 2014). Additionally, the GSK3 inhibitor Lithium Chloride, which has long been used to treat patients with bipolar disorder, has never been linked to increased cancer incidence (McCurbey et al., 2014). In fact, it has been observed that GSK3 inhibitors can decrease cellular proliferation of some cancers, including melanoma, prostate, and pancreatic tumors (Miyashita et al., 2009; Flaherty et al., 2012). GSK3 inhibitors may eventually be used as an anticancer treatment (McCurbey et al., 2014); the GSK3 inhibitor LY2090314 is currently being tested in clinical trials for the treatment of metastatic pancreatic cancer [NCT01632306] and acute leukemia [NCT01214603].

10. Conclusions

Recent studies have led to the emerging concept that when the MiT members MITF, TFEB or TFEC are amplified or overexpressed in melanoma, renal cell carcinomas, clear cell sarcomas, and perhaps other cancers. The trafficking of lysosomal/autophagosome organelles and their function in proteostasis should be significantly altered. In the case of MITF overexpression vesicular trafficking reprogramming increases MVB/late endolysosomes that
enhance canonical Wnt signaling by sequestering destruction complex components, compromising lysosomal/autophagy functions, and impair oncogene-induced senescence, conferring a growth advantage to tumor cells. Wnt-driven stabilization of proteins (Wnt/STOP) should be potentiated in these tumors, with many cellular proteins (in addition to β-Catenin) becoming more stable. In melanomas, MITF itself is stabilized, giving rise to a positive feedback regulation (Fig. 5-8) that further affects endolysosomal trafficking and Wnt signaling during the proliferative stages of melanoma growth (Ploper et al., 2015). One possibility is that this regulatory loop might be manipulated via GSK3 inhibitors to entice differentiation (through phenotype-switching caused by increased MITF levels) (Sáez-Ayala et al., 2013; Wellbrock and Arozarena, 2015) of the slowly dividing stem-like cells that make melanomas so refractory to treatment.

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CONCLUDING REMARKS:

Some synthesis of work in Xenopus patterning led to the study of integration of signaling pathways. This thesis first investigated the fine integration of the Chordin-BMP pathway. In the embryo, canonical Wnt signaling is also integrated with BMP leading to the discovery that up to 20% of cellular proteins may be possibly stabilized by Wnt. I chose one protein, MITF, which was previously described as a melanoma oncogene and found out that not only was it stabilized by Wnt signaling, but also that it drives endolysosomal biogenesis. This provides a hypothesis for the oncogenic mechanism of the MITF family of transcription factor oncogenes.


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