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Targeting Cas Family Proteins as a Novel Treatment for Breast Cancer

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1. Introduction

Frequently, breast cancer is treated before and after surgery with chemotherapy, hormone, and radiation therapies. However, breast cancers can evolve and stop responding to chemotherapeutic drugs, including adriamycin (doxorubicin), and hormone therapy with tamoxifen. A new generation of targeted biological agents demonstrates a high effectiveness at lower toxicity. Treatment with these specific drugs is limited to subsets of breast cancers that depend on their targets, and eventually patients develop resistance to these drugs as well. This strongly indicates the need to develop novel approaches to fight breast tumor cells and to prevent or reduce drug-resistance. The Cas family of proteins play significant roles in development, proliferation, cell cycle control, cell survival, migration, and invasion. Some of its members, in particular p130^{Cas}/*BCAR1*, has been implicated with tamoxifen as well as adriamycin resistance in mammary tumors. Here we review the role of the Cas family of proteins in breast cancer and summarize the potential development of anti-cancer therapeutics targeting this important family of adapter-type proteins.

1.1 Cas family

Proteins of the Cas (Crk-associated substrate) family function as scaffolds for large multi-protein complexes that integrate the response to numerous stimuli including growth factors, integrin engagement, and hormone release (Tikhmyanova *et al.* 2010; Bouton *et al.* 2001). Cas family members comprise p130^{Cas}/*BCAR1* (Sakai *et al.* 1994a; Brinkman *et al.* 2000), HEF1 (also known as *NEDD9*, CASS2, Cas-L) (Law *et al.* 1996; Minegishi *et al.* 1996), Efs/Sin (CASS3) (Alexandropoulos & Baltimore 1996; Ishino *et al.* 1995), and CASS4 (HEPL) (Singh *et al.* 2008). p130^{Cas} is the founding member and was first identified as a major tyrosine-phosphorylated 130 kDa protein in cells transformed by the v-crk and v-src oncogenes (Sakai *et al.* 1994a). HEF1 (human enhancer of filamentation)/*NEDD9* (neural precursor cell expressed, developmentally downregulated 9) was isolated in a screen for human proteins that confer morphoregulatory changes leading to filamentous budding in yeast *Saccharomyces cerevisiae* (Law *et al.* 1996). In addition, HEF1 was independently isolated based on its homology to p130^{Cas} (Minegishi *et al.* 1996).

Efs/Sin (embryonal Fyn-associated substrate/Src-interacting protein) was identified as a protein binding to the Src-homology (SH) 3 domain of Fyn (Alexandropoulos & Baltimore 1996; Ishino *et al.* 1995). Most recently, the fourth member CASS4 (Cas scaffolding protein family member 4)/HEPL (HEF1-Efs-p130^{Cas}-Like) was identified using reiterative BLAST

analysis for protein and mRNA sequences of Cas family members (Singh *et al.* 2008). The expression patterns of Cas family members are distinct. p130^{Cas} is ubiquitously expressed in adult tissues, suggesting that it plays an essential role in normal cell physiology (Defilippi *et al.* 2006; Sakai *et al.* 1994a). HEF1 is found primarily in lymphocytes and in lung and breast epithelium (Law *et al.* 1996; Law *et al.* 1998; Minegishi *et al.* 1996). Highest levels of Efs/Sin and CASS4 expression are found in the placenta and brain (Ishino *et al.* 1995), and lung and spleen (Singh *et al.* 2008), respectively.

By facilitating the interaction of Src family kinases (SFKs), focal adhesion kinase (FAK), and recruiting the adaptor proteins Crk and Nck, members of the Cas family play significant roles in signaling networks involved in cell survival (Cabodi *et al.* 2006; Kim *et al.* 2004), cell cycle regulation (Law *et al.* 1998; Ma *et al.* 2007; Yamakita *et al.* 1999), proliferation, and invasion (Huang *et al.* 2002; Klemke *et al.* 1998) (as depicted in Figure 1). These cellular programs are frequently deregulated in different cancer types (Cabodi *et al.* 2010a; Henderson & Feigelson 2000; Marcotte & Muller 2008; Hanahan & Weinberg 2011) and concordantly members of the Cas family have been extensively associated with the development and progression of different tumors in particular mammary carcinomas as reviewed in Section 2.

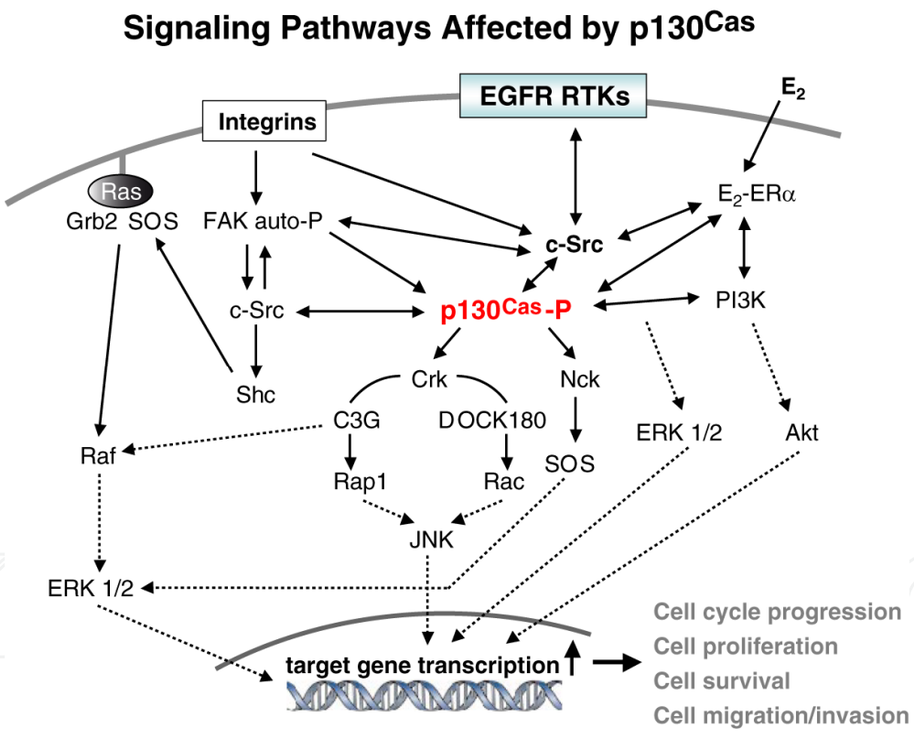

Fig. 1. Major signaling pathways affected by p130^{Cas}. Integrin engagement, growth factor-mediated activation of receptor tyrosine kinases (RTKs), and estrogen (E_2)-induced non-genomic estrogen receptor (ER) alpha signaling results in tyrosine phosphorylation of p130^{Cas}. The phosphorylated/activated p130^{Cas} recruits various effector proteins, thereby generating a signaling node, that activates downstream pathways leading to the induction of transcriptional programs promoting cell cycle progression, proliferation, survival, and migration/invasion. Solid lines depict direct interactions; dashed lines show pathways that have additional steps in between.

1.2 The domain structure of Cas family proteins

Cas proteins exhibit a highly conserved modular domain structure and vary from 561 to 870 amino acids (Tikhmyanova *et al.* 2010). To date no evidence has been found for intrinsic enzymatic activity of the Cas family members. The members are characterized by multiple protein-protein interaction domains including an amino-terminal SH3 domain, a central substrate domain (SD) containing multiple tyrosine phosphorylation sites, a serine-rich region (SER), and a carboxy-terminal domain (CTD) containing a bi-partite Src-binding motif (SBM) (Figure 2). Cas proteins have numerous binding partners for each domain which are summarized in Table 1.

Domain	Interacting partners	Reference
SH3 domain	C3G	(Kirsch *et al.* 1998)
	CMS/CD2AP	(Kirsch *et al.* 1999)
	CIZ	(Nakamoto *et al.* 2000)
	FAK	(Polte & Hanks 1995; Law *et al.* 1996; Singh *et al.* 2008)
	FRANK	(Harte *et al.* 1996)
	PR-39	(Chan & Gallo 1998)
	PTP-1B	(Liu *et al.* 1996)
	PTP-PEST	(Garton *et al.* 1997)
	Pyk2	(Astier *et al.* 1997; Lakkakorpi *et al.* 1999)
Substrate Domain	Crk family: CrkI, CrkII, CrkL	(Burnham *et al.* 1996; Ishino *et al.* 1995; Petruzzelli *et al.* 1996; Sakai *et al.* 1994a; Salgia *et al.* 1996)
	Nck	(Schlaepfer *et al.* 1997)
	SHP-2	(Prasad *et al.* 2001; Yo *et al.* 2009)
	c-Src	(Shin *et al.* 2004)
Serine-rich Domain	14-3-3	(Briknarova *et al.* 2005)
Carboxy-terminal domain	AIP4	(Feng *et al.* 2004)
	APC/C, CDH1	(Nourry *et al.* 2004)
	Bmx/Etk	(Abassi *et al.* 2003)
	NSP family: BCAR3/NSP2, NSP1, CHAT	(Gotoh *et al.* 2000; Lu *et al.* 1999; Sakakibara & Hattori 2000)
	Nephrocystin	(Donaldson *et al.* 2000)
	$p130^{Cas}$, HEF1, Id2	(Law *et al.* 1999)
	p140Cap	(Di Stefano *et al.* 2004)
	PI3K	(Li *et al.* 2000)
	Src family: c-SRC, LCK, LYN, HCK, FYN, YES	(Alexandropoulos & Baltimore 1996; Ishino *et al.* 1995; Kanda *et al.* 1999; Nakamoto *et al.* 1996; Nasertorabi *et al.* 2006; Nishio & Suzuki 2002; Pellicena & Miller 2001; Singh *et al.* 2008)
Not mapped/ indirect	DDR	(Shintani *et al.* 2008)
	ER alpha	(Cabodi *et al.* 2004)
	Grb2	(Wang *et al.* 2000)
	Smad3	(Liu *et al.* 2000)

Table 1. Interacting partners of Cas family proteins.

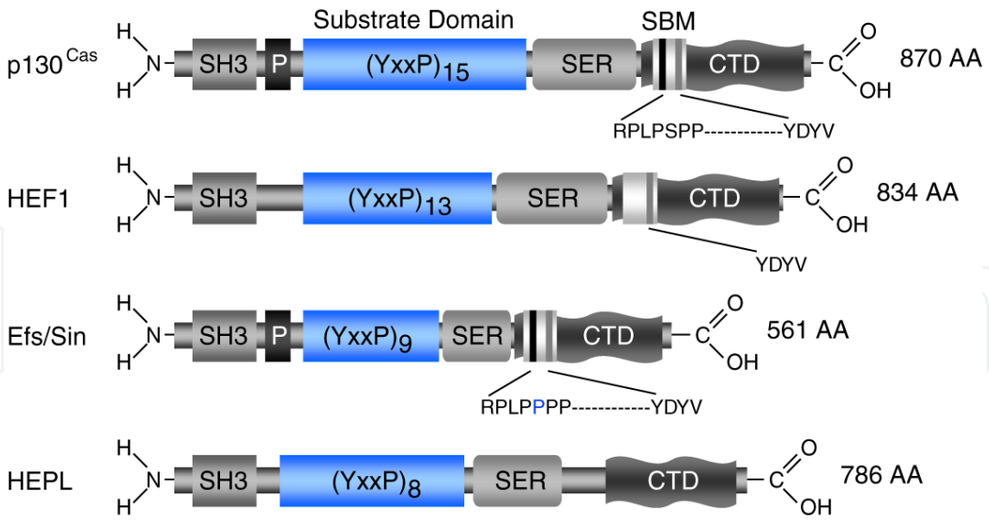

Fig. 2. Domain structure of the Cas family members. SH3, Src homology 3 domain. P, proline-rich region. SER, serine-rich domain. SBM, bi-partite Src-binding motif. CTD, carboxy-terminal domain. The number of YxxP motifs, representing SH2 domain binding sites when phosphorylated, are indicated.

1.2.1 The SH3 domain

The amino-terminal SH3 domain is an interaction module that associates with proteins containing proline-rich motifs with the core consensus sequence PxxP (Ren *et al.* 1993). The SH3 domain of p130^{Cas} selects binding sites sharing the consensus motif *XX*Pp+Pp*X* (where + and *X* represent positively charges and non-conserved residues, respectively; lower case positions contain residues that tend to be proline) (Kirsch *et al.* 1998). The specificity of the p130^{Cas} SH3 domain is strongly dependent on the positively charged amino acid in the position P_2 (see nomenclature in Yu *et al.* 1994). The functional relevance of the interaction of Cas family members with FAK via the Cas SH3 domain has been extensively studied (Polte & Hanks 1995; Parsons *et al.* 2000; Law *et al.* 1996; Singh *et al.* 2008; Provenzano & Keely 2009; Tikhmyanova *et al.* 2010). Utilizing p130^{Cas} SH3 domain deletion mutants, studies indicated that this domain and its interaction with FAK are necessary for phosphorylation and the localization of p130^{Cas} to focal adhesions (FAs) (Nakamoto *et al.* 1997). More recent studies, further explored the temporal and spatial involvement of p130^{Cas} in FA dynamics and showed the influence of p130^{Cas} in FA turnover and controlling the migratory response (Donato *et al.* 2010; Meenderink *et al.* 2010). The p130^{Cas} SH3 domain was shown to be necessary for tyrosine phosphorylation of the SD and the promotion of cell migration (Donato *et al.* 2010), in conditional FAK-deficient mammary tumor cells a reduction in the phosphorylation of Y249 within the p130^{Cas} SD was observed (Provenzano *et al.* 2008). These studies uncovered the significance of the Cas SH3 domain in the spatial regulation of Cas protein function and in particular its role for the phosphorylation of the SD of the Cas proteins (as described in the next section).

1.2.2 The substrate domain (SD)

The SD region of the Cas family members is situated adjacent to the SH3 domain and contains clusters of YxxP tyrosine phosphorylation sites (Figure 2). The SD of p130^{Cas} and

HEF1 contain 15 and 13 YxxP motifs, whereas only eight and nine of these motifs are present in Efs/Sin and HEPL, respectively (Alexandropoulos & Baltimore 1996; Ishino *et al.* 1995; Law *et al.* 1996; Sakai *et al.* 1994a), which upon phosphorylation by Src family members recruit small adaptor proteins such as Crk, CRKII, CrkL, and Nck via their respective SH2 domains (Burnham *et al.* 1996; Harte *et al.* 1996; Minegishi *et al.* 1996).

The SFKs, in particular c-Src, play significant roles in the activation of Cas proteins by phosphorylation of tyrosine residues within the SD, resulting in coupling to downstream effector molecules (Sakai *et al.* 1997; Schlaepfer *et al.* 1997). Correspondingly, c-Src-deficient cells show reduced Cas phosphorylation levels. Furthermore, *in vitro* kinase assays show that c-Src, in comparison to FAK, has a stronger ability to phosphorylate the SD of $p130^{Cas}$ (Ruest *et al.* 2001).

In order to prevent permanent activation of Cas-related pathways, the protein tyrosine phosphatases (PTPs) PTP-1B (Liu *et al.* 1996), PTP-PEST (Garton *et al.* 1997; Cote *et al.* 1998), and leukocyte antigen related (LAR)-PTP (Hoon *et al.* 2003) can be recruited to Cas family members to dephosphorylate Cas proteins and other associated molecules. This results, for instance, in the subsequent cleavage and degradation of the $p130^{Cas}$ protein (Hoon *et al.* 2003; Weng *et al.* 1999) and/or the disassembly of signaling complexes in FAs (Angers-Loustau *et al.* 1999).

The tyrosine phosphorylation/activation status of Cas family members is altered by diverse stimuli, including environmental influences such as growth factors, integrin, and estrogen signaling as well as intrinsic signals, thereby modulating multiple signal transduction networks as depicted in Figure 1 (reviewed in Bouton *et al.* 2001; Cabodi *et al.* 2010a; Tikhmyanova *et al.* 2010). SD phosphorylation correlates with transformation and the effector signaling pathways relevant in breast cancer development and progression involving the Cas family proteins are reviewed in Section 2.

1.2.3 The serine-rich domain (SER)

This domain is situated between the SD and the CTD and is enriched in serines and threonines (Alexandropoulos & Baltimore 1996; Law *et al.* 1996; Sakai *et al.* 1994a; Sakai *et al.* 1994b; Singh *et al.* 2008). Although the SER was initially thought to separate and orient the SD and SBM, several studies identified important properties. During mitosis an increase in the phosphorylation of serine and threonine residues of $p130^{Cas}$ has been observed (Yamakita *et al.* 1999). Notably, adhesion-dependent serine phosphorylation of $p130^{Cas}$ is associated with an invasive phenotype in breast cancer cells (Makkinje *et al.* 2009). The solution structure of the SER of $p130^{Cas}$ was determined by nuclear magnetic resonance spectroscopy revealing that it folds as a four-helix bundle. Site-directed mutagenesis and binding assays characterized this domain as an interaction site for 14-3-3 proteins (Briknarova *et al.* 2005). Proteins of the 14-3-3 family act as chaperones or scaffolds and are involved in signaling, cell cycle control, and apoptosis (reviewed in Bridges & Moorhead 2004).

1.2.4 The carboxy-terminal domain (CTD)

Although the CTD, which folds as a helix-loop-helix (HLH) structure, is the most conserved region among Cas family members, one of the striking differences regarding this domain is the presence or absence of the bi-partite SBM. The SBM consists of a proline-rich motif (RPLP S/P PP) that interacts with the SH3 domain of SFKs and a YDYVHL motif that, when

phosphorylated, interacts with the SH2 domain of SFKs. This region is present in p130Cas and Efs/Sin but absent in CASS4 (Alexandropoulos & Baltimore 1996; Nakamoto *et al.* 1996; Singh *et al.* 2008; Nasertorabi *et al.* 2006). HEF1 contains the SH2 binding motif but lacks the SH3 binding motif (Law *et al.* 1996). This may influence the ability of HEF1 to bind SFKs and to become phosphorylated, since amino acid substitutions in the RPLP S/P PP sequence reduces/abolishes binding to Src (Burnham *et al.* 1999; Burnham *et al.* 2000; Nakamoto *et al.* 1996). However, TGF-β-mediated tyrosine phosphorylation of HEF1 (Zheng & McKeown-Longo 2002) and attachment-induced tyrosine phosphorylation of CASS4 (Singh *et al.* 2008), are both dependent on Src kinase activity. It has been suggested that additonal mechanisms may regulate this modification such as FAK-dependent recruitment of c-Src to p130Cas (Ruest *et al.* 2001). The differences in the SBM of Cas proteins likely indicate distinct functions among the Cas proteins, which are highly dependent on the phosphorylation of the SD by SFKs and represents an important event in different cellular programs (see 1.2.2).
The CTD along with the SH3 domain target p130Cas to FAs, as deletion of the CTD prevents the localization of p130Cas to FAs (Harte *et al.* 2000). In addition, the CTD mediates the homo- and heterodimerization of p130Cas and HEF1 (Law *et al.* 1999) thereby potentially generating additional regulatory mechanisms.

2. Involvement of Cas family members in mammary carcinomas

Over the last decade *in vivo* studies in different organisms and *in vitro* studies in cells in culture accumulated evidence that individual Cas family members play central roles in the development and progression of mammary carcinomas. The p130Cas and HEF1 proteins are the best studied in this context. Primary breast tumors contain elevated p130Cas levels, which correlate with increased rate of relapse and with poor response to tamoxifen treatment (van der Flier *et al.* 2000). Increased p130Cas expression was also found in tumor cells isolated from pleural effusions of breast cancer patients in comparison to primary tumors (Konstantinovsky *et al.* 2010). In feline and canine breast cancers the levels of p130Cas positively correlate with advanced breast disease as well (Scibelli *et al.* 2003). Furthermore, our *in vitro* studies have shown increased p130Cas levels in the tamoxifen resistant breast cancer cells TAM-R (Soni *et al.* 2009), which were derived from tamoxifen sensitive MCF-7 cells (Knowlden *et al.* 2003).
In 2000, Brinkman and colleagues identified the gene of p130Cas, in a retroviral insertion screen as a factor that mediates resistance to tamoxifen in breast cancer cell lines (Brinkman *et al.* 2000; Dorssers *et al.* 1993). Subsequently, the gene located on chromosome 16q23.1 was named *BCAR1* (breast cancer anti-estrogen resistance 1). Although HEF1 has a similar domain structure it was unable to support long-term anti-estrogen resistant cell proliferation (Brinkman *et al.* 2009). Chimeric p130Cas/HEF1 proteins generated by exchange of defined domains, identified the SD of p130Cas as the region contributing to anti-estrogen resistance in breast cancer cells (Brinkman *et al.* 2009). Accordingly, disruption of the p130Cas signaling node by ectopic expression of an isolated constitutively tyrosine phosphorylated SD of p130Cas in the cytoplasm (as described in detail in Section 3) led to reduced proliferation and re-sensitization of tamoxifen resistant breast cancer cells to tamoxifen (Kirsch *et al.* 2002; Soni *et al.* 2009).
The mechanisms by which Cas proteins may promote mammary carcinomas and acquired tamoxifen resistance are manifold and under extensive investigation. It has been shown that estrogen treatment triggers the rapid and transient association of p130Cas with the estrogen

receptor (ER) alpha in the cytoplasm, thus mediating non-genomic ER signaling in human breast cancer cells (Cabodi *et al.* 2004). This is dependent on c-Src activation and results in the formation of a multi-molecular complex containing p130^{Cas}, c-Src, and the p85 subunit of phosphatidylinositol 3-kinase (PI3K) and subsequent activation of extracellular-signal regulated kinase (ERK) 1/2. Importantly, overexpression of p130^{Cas} as well as short-interfering (si) RNA-mediated reduction of p130^{Cas} experiments in T47D breast cancer cells indicated that p130^{Cas} enhances the estrogen-dependent Src and Erk1/2 activities (and accelerates the kinetics in response to stimulation) (Cabodi *et al.* 2004). Long-term treatment of estrogen-dependent mammary carcinoma cells with the estrogen antagonist tamoxifen led to increased phosphorylation levels of p130^{Cas} (Cowell *et al.* 2006; Soni *et al.* 2009), suggesting that anti-estrogens modulate intrinsic mechanisms to deregulate Cas protein function.

Resistance to the anti-estrogens tamoxifen and fulvestrant, is associated with enhanced growth factor signaling involving the upregulation of epidermal growth factor receptor (EGFR) family and alteration of the AKT signaling pathway (Knowlden *et al.* 2003; Soni *et al.* 2009; Zhang *et al.* 2009; Frogne *et al.* 2009). Consistently, interference with p130^{Cas} signaling results in the attenuation of the ERK and PI3K/Akt survival pathways in breast cancer cells (Soni *et al.* 2009). Moreover, overexpression of p130^{Cas} mediates resistance to the chemotherapeutic drug adriamycin in mammary tumor cells by activating c-Src, Akt, and ERK1/2 growth and survival pathways (Ta *et al.* 2008).

More recent *in vivo* studies in transgenic mice overexpressing p130^{Cas} in mammary epithelial cells, showed substantial mammary epithelial cell hyperplasia during development and pregnancy, and delayed involution (Cabodi *et al.* 2006). Activation of Src, ERK1/2, mitogen-activated protein kinase (MAPK), and Akt pathways contribute to these phenotypes by inducing proliferation and inhibiting apoptosis.

Importantly, accelerated mammary tumor formation has been observed in double transgenic mice that overexpress both p130^{Cas} and the activated form of HER2/neu (human epidermal growth factor receptor 2) compared to the HER2/neu single transgenic mice without p130^{Cas} (Cabodi *et al.* 2006). Delivery of p130^{Cas}/*BCAR1*-specific siRNAs into the mammary gland of transgenic BALB–HER2/neu mice carrying the activated *HER2/neu* oncogene was sufficient to inhibit HER2/neu signaling and decreased the growth of spontaneous tumors *in vivo* (Cabodi *et al.* 2010b).

The balance between canonical and noncanonical transforming growth factor (TGF)-β signaling in mammary carcinomas is also regulated by p130^{Cas} (Wendt *et al.* 2009). Maintaining this balance is critical as TGF-β acts as both a tumor-suppressor or tumor-promoter depending on the tumor microenvironment and tumor stage (as reviewed in Ikushima & Miyazono 2010; Meulmeester & Ten Dijke 2011). Forced expression of either full length p130^{Cas} or the CTD of p130^{Cas} in mammary epithelial cells (MECs) shifted TGF-β signaling from Smad2/3 to p38 MAPK activation resulting in resistance of TGF-β-induced growth arrest and increased invasion and metastasis of MECs *in vivo* utilizing an orthotopic mouse model (Wendt *et al.* 2009).

In addition to p130^{Cas}, HEF1, also mediates TGF-β tumor promoting activities. TGF-β signaling upregulates HEF1 thereby enhancing mammary carcinoma cell scattering and the transition from collective cell motility to single cell motility (Giampieri *et al.* 2009). Similar to the TGF-β study, HEF1 overexpression in MCF-7 breast cancer cells increases the migration and invasion *in vitro* (Fashena *et al.* 2002). In MMTV-polyoma virus middle T antigen

(PyMT) mice crossed with the HEF1-deficient (HEF$^{-/-}$) mice delayed mammary tumor formation and reduced tumor incidence was observed (Izumchenko *et al.* 2009). Most of the mammary tumors excised from PyMT/HEF1$^{-/-}$ mice showed reduced activation of AKT, FAK, Src, and ERK1/2 compared to HEF1 wildtype animals. In contrast, an siRNA screening approach to identify genes that regulate migration in non-transformed mammary epithelial MCF-10A cells demonstrated an inhibitory function of HEF1 on migration (Simpson *et al.* 2008). Furthermore, HEF1 is part of a lung metastasis signature for primary breast cancers (Minn *et al.* 2005). In this study, an orthotopic MDA-MB-231 breast cancer mouse model was used and HEF1 was found to be down-regulated in highly lung metastatic mammary cancer cells. These results may suggest, that high levels of HEF1 contribute to early stages of breast cancer and a loss of expression during tumor progression may promote later stages leading to metastases formation of tumor cells.
In summary, the studies reviewed here indicate the extensive involvement of Cas family members, specifically p130^{Cas} and HEF1, in the transformation of mammary epithelium as well as acquired resistance of breast cancers to several therapeutic agents. The effects of the Cas proteins might be further amplified by simultaneous and synergistic activation of multiple signaling effector pathways, in particular down-stream of the SD of p130^{Cas} and HEF1.

2.1 Role of Cas SD effector protein signaling in mammary carcinomas

As described above, phosphorylaion of the Cas SD and subsequent coupling to effector molecules has been implicated in the transformation of cells, breast cancer progression, and acquired tamoxifen resistance (reviewed in Tikhmyanova *et al.* 2010). To better understand how the SD of the Cas proteins may contribute to these malignant processes the involvement of SD-interacting proteins in breast cancer is reviewed in this section.

2.1.1 The Crk family

Members of the Crk (chicken tumor virus no. 10 regulator of kinase) family, consisting of CRKI, CRKII, generated by alternative splicing of transcripts of the *CRK* gene, and CRKL (CRK-like protein) are SH2 and SH3 domain containing adaptor proteins (Matsuda *et al.* 1992; ten Hoeve *et al.* 1993; Feller & Lewitzky 2006). The Crk family SH2 domains bind to p130^{Cas} upon phosphorylation of the SD and recruit additional downstream effectors via their SH3 domains. The amino-terminal SH3 domain of Crk binds to guanine nucleotide exchange factors (GEFs), including C3G, Sos (Son of sevenless) (Feller *et al.* 1995; Okada & Pessin 1996), and DOCK1 (dedicator of cytokinesis 1, also known as DOCK180) (Hasegawa *et al.* 1996). This complex leads to the activation of the small GTPases Rap1 (Gotoh *et al.* 1995) and Rac (Dolfi *et al.* 1998), and subsequently JNK (c-Jun N-terminal kinase) signaling (shown in part in Figure 1) (Dolfi *et al.* 1998).
Crk proteins have been associated with several different tumor types and especially with the promotion of an invasive phenotype and cell migration (Cabodi *et al.* 2010a; Tikhmyanova *et al.* 2010). Interestingly, Klemke's group was the first to reveal that p130^{Cas}-crk coupling is involved in HER2/neu -mediated cell migration (Spencer *et al.* 2000). Subsequently, Park's group found elevated CrkI and CrkII protein levels in human mammary tumors and showed that siRNA-mediated CrkI/II knockdown results in a significant decrease in migration and invasion of breast cancer cells (Rodrigues *et al.* 2005). More recently, the same group observed in post-pubertal MMTV-CrkII transgenic mice premature ductal branching

that was associated with increased proliferation (Fathers *et al.* 2010). The mammary tumor incidence in MMTV-CrkII mice was 17.6% compared to 4% in female control mice with a similar latency of approximately 15 months. While Crk has been shown to induce cell migration, no metastatic lesions were found in any of the MMTV-CrkII animals. This suggests that CrkII plays a more important role at the early stages of breast carcinomas *in vivo*.

2.1.2 The Src-family tyrosine kinases (SFKs)

The SFKs are comprised of ten members of which c-Src, Lck, Lyn, Hck, Fyn, and Yes phosphorylate the SD of the Cas proteins (Alexandropoulos & Baltimore 1996; Ishino *et al.* 1995; Kanda *et al.* 1999; Nakamoto *et al.* 1996; Nasertorabi *et al.* 2006; Nishio & Suzuki 2002; Pellicena & Miller 2001; Singh *et al.* 2008). In addition to phosphorylating the $p130^{Cas}$ SD, c-Src binds to phosphorylated tyrosine residues within SD *in vitro* (Shin *et al.* 2004).

SFKs have a conserved structure containing SH1 (kinase), SH2, SH3, and SH4 (membrane targeting) domains and transactivate interacting partners by phosphorylation resulting in the activation of multiple signaling pathways as presented in part in Figure 1 (reviewed in Mayer & Krop 2010; Wheeler *et al.* 2009). The kinase activity is tightly regulated by tyrosine-phosphorylation on the carboxyl terminus by CSK (c-src tyrosine kinase) resulting in intramolecular binding and an inactive closed conformation (Superti-Furga *et al.* 1993). The precise cellular regulation of c-Src is of major relevance and $p130^{Cas}$ has been postulated to activate c-Src by disrupting the intramolecular inactive conformation (Nasertorabi *et al.* 2006; Burnham *et al.* 2000).

SFKs are central players in multiple cellular programs that are often dysregulated during tumorigenesis and progression and several of the members have been associated with malignant transformation. The founding member c-Src was the first proto-oncogene to be sequenced in the early 1980s (Czernilofsky *et al.* 1980; Schwartz *et al.* 1983; Takeya & Hanafusa 1982) and over the past 30 years several therapeutic agents to inhibit Src kinase activity have been developed and clinical trials are ongoing (summarized in Aleshin & Finn 2010; Mayer & Krop 2010).

Several studies have demonstrated the relevance of Src in breast cancer as enhanced expression and activity of c-Src was observed in human mammary carcinoma cell lines and tumor tissues (Biscardi *et al.* 1998; Jacobs & Rubsamen 1983; Ottenhoff-Kalff *et al.* 1992; Verbeek *et al.* 1996). Studies by Muller's group unequivocally showed that expression of an activated c-Src protein in the mammary gland of mice induces tumor formation, though with long latency (Webster *et al.* 1995). Importantly, tumor formation in transgenic mice expressing the PyMT oncogene under the control of the MMTV promoter is c-Src dependent as PyMT-Src-deficient mice rarely developed mammary tumors, whereas a more rapid tumor progression was found in the MMTV-PyMT control mice (Guy *et al.* 1994). Furthermore, aberrated c-Src expression and activity has been associated with Her2/neu transformation *in vivo* (Muthuswamy & Muller 1995). Several SFK specific inhibitors have been developed which suppress migration and invasion of human breast cancer cells (Vultur *et al.* 2008). These compounds also reduce the incidence of metastasis formation after intracardiac injection of human breast cancer cells in nude mice (Rucci *et al.* 2006).

2.1.3 SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase)

Through its interactions with numerous proteins via its two SH2 domains, the protein tyrosine phosphatase SHP-2 regulates oncogenic transformation (as reviewed in Matozaki *et*

al. 2009). Conflicting results have been reported for a role of SHP-2 in mammary adenocarcinomas in regard to the effect on migration. Upon tyrosine phosphorylation of HEF1, SHP-2 associates with the HEF1 SD and dephosphorylates it thereby inhibiting HEF1-mediated cell migration (Yo *et al.* 2009). Conversely, SHP-2 acts as a positive regulator of migration of MCF7 breast cancer cells *in vitro* and promotes metastasis of MCF7 cells, when injected into the abdominal cavity of nude mice *in vivo* (Wang *et al.* 2005).

2.1.4 The Nck family

Similar to the Crk proteins, the two Nck family members are SH2/SH3 domain-containing adapters which regulate tyrosine kinase signaling (reviewed in Buday *et al.* 2002). Nck was first identified in a screen of a human melanoma cDNA library using antibodies against the melanoma cell adhesion molecule (MCAM) (Lehmann *et al.* 1990). In MCF-7 breast cancer cells, Nck is required for fibroblast growth factor (FGF)-2-induced DNA synthesis (Liu et al. 1999). Furthermore, Nck facilitates invadopodia formation and extracellular matrix (ECM) degradation in various tumor cell lines including breast cancer cells (Stylli et al. 2009).

These studies summarized here emphasize the importance of Cas effector proteins in the promotion of breast cancer *in vitro* and *in vivo*. They highlight the potential importance of targeting the p130^{Cas} signaling node in human breast cancers, as Cas family members might contribute to this malignancy through their association with these SD interacting molecules.

3. Novel strategies to develop therapeutic agents for targeting Cas signaling in mammary carcinomas

Though many studies have suggested that p130^{Cas} and HEF1 are critical for phenotypic changes that drive breast cancer progression and metastasis (see Section 2), no therapeutic agents (drugs) have been developed that target these important proteins. It might be feasible to inactivate cancer promoting adaptor proteins by several mechanisms among them (a) downregulating their expression or (b) by interfering with specific protein-protein interaction modules. Defillipi's group used an RNAi-based approach to mediate downregulation of p130^{Cas} by intranipple injection of siRNA resulting in a reduction of tumor growth in BALB-HER2/neu mice (Cabodi *et al.* 2010b). These results are very promising and may warrant further exploration.

Current research has now revealed a clearer picture regarding the functions of the different domains in p130^{Cas} signaling and tumor formation (see Sections 1 and 2). Therefore, inhibitors targeting individual domains, such as the SD, SH3, and/or CTD domain, might confer additional specificity and maintain the functional properties of the other domains thereby reducing potential adverse effects. As an alternative approach, the Src*/CasSD decoy molecule (Kirsch et al., 2002 and summarized in Section 3.1) could be used as a starting point to develop inhibitors that target only certain Cas functions.

3.1 Blocking p130^{Cas} SD signaling by utilization of a phosphorylated p130^{Cas} SD

As discussed above, the important role for p130^{Cas} in breast cancer has been demonstrated by several groups, with a critical function for the SD emerging in cell transformation. Kirsch and colleagues previously investigated the role of the Cas SD in transformation by functionally separating Cas from upstream signals (Kirsch *et al.* 2002). The p130^{Cas} SD was fused to the Src kinase domain with attenuated activity [activating tyrosine 416 replaced

with phenylalanine (Y416F); designated as Src*/CasSD] (Figure 3) (Kmiecik & Shalloway 1987; Piwnica-Worms *et al.* 1987). As controls, a Src kinase inactive mutant (K295M) (Jove *et al.* 1987) fused to the p130^{Cas} SD (Src^{KM}/CasSD) and the isolated Src* were employed (Kirsch *et al.* 2002). The initial hypothesis, that this constitutively phosphorylated chimera would act as dominant active molecule by circumventing upstream signaling had to be revised as the results obtained in transient and stably expressing cell systems revealed a dominant negative effect on downstream signaling. It was subsequently found that the Src*/CasSD chimera attenuated cellular transformation. Expression of Src*/CasSD resulted in a significant reduction of colony formation of v-crk transformed NIH3T3 cells in soft agar assays. Further experiments, suggested that the isolated tyrosine phosphorylated CasSD acts as a decoy for v- and c-Crk thereby blocking the ability of v-crk to transform these cells involving a reduction in JNK activation (Kirsch *et al.* 2002).

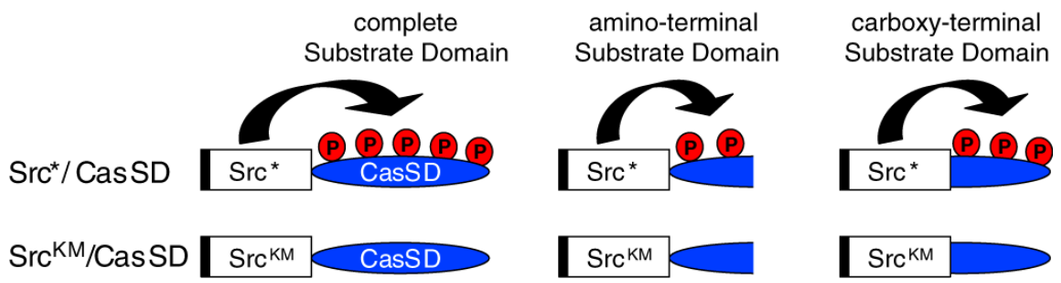

Fig. 3. Representation of the dominant negative p130^{Cas} (Src*/CasSD) and control (Src^{KM}/CasSD) fusion constructs generated by Kirsch and colleagues, 2002. Src*, attenuated Src kinase domain. Src^{KM}, inactive Src kinase domain. CasSD, substrate domain of p130^{Cas}. P, phosphorylation.

Additional studies utilizing this approach in different cellular contexts revealed proof of principle of the Src*/CasSD decoy approach. In BxPC3 pancreatic adenocarcinoma cells the Src*/CasSD expression prevented collagen I-mediated upregulation of N-cadherin and cell scattering (Shintani *et al.* 2008). In tamoxifen resistant breast cancer cells TAM-R, expression of the chimeric molecule attenuated several signaling pathways involved in breast carcinoma progression and acquired tamoxifen resistance (see below) (Soni *et al.* 2009).
TAM-R cells were established by long-term exposure of estrogen-dependent MCF-7 cells to tamoxifen (Hiscox *et al.* 2004). Importantly, similar to anti-estrogen resistant breast cancers, endogenous p130^{Cas} levels are increased and highly phosphorylated in these cells (Soni *et al.* 2009), a further indication that p130^{Cas} contributes to tamoxifen resistance. A major finding of our study investigating the effects of the inhibition of p130^{Cas} by the Src*/CasSD chimera on breast cancer cells was the re-sensitization of TAM-R cells to tamoxifen resulting in increased apoptosis (Soni *et al.* 2009). Of note, the Src*/CasSD induced apoptosis was specific for TAM-R cells and not detected in the parental MCF-7 cells expressing lower p130^{Cas} levels. Moreover, expression of Src*/CasSD resulted in reduced cell numbers of MCF-7 and TAM-R cells and in a reversion to a more epithelial-like phenotype in TAM-R cells. In TAM-R cells, these observations were accompanied by elevated levels of ERα, E-cadherin stabilization at cell-cell boundaries, and reduced migration and consistently, enhanced cell clustering. Furthermore, employment of the Src*/CasSD approach in TAM-R cells led to the reduction of growth factor signaling as seen by an attenuated PI3K/AKT pro-survival pathway, and a reduced activation of the MAPK/ERK pathway (Soni *et al.* 2009).

Taken together, these studies suggest, that blocking endogenous p130^{Cas} function by ectopic expression of a constitutively phosphorylated p130^{Cas} SD may represent an important tool not only for further elucidating the mechanisms by which Cas proteins contribute to breast cancer and other malignancies but also to develop potential therapeutic agents targeting this domain as discussed below.

3.2 The Src*/CasSD decoy approach - a starting point to develop potential therapeutics for breast cancer

The Src*/CasSD approach implies at least two different strategies to inhibit downstream signaling of Cas family proteins: **1.** Develop therapeutic agents reflecting the structure of important parts of the phosphorylated SD (mimetic), which act as a decoy for SD interacting molecules, thereby competing with endogenous Cas proteins for binding partners; **2.** Design compounds that bind to functionally relevant tyrosine motifs in the SD of the endogenous Cas proteins to block the recruitment of SD binding adapter proteins. **3.** Design drugs that bind to the SH2 domains of the interacting proteins as it has been elegantly demonstrated for Grb2 (Growth factor receptor-bound protein 2) inhibitors (Atabey *et al.* 2001; Dharmawardana *et al.* 2006). The advantage of the first two approaches lies in the fact that several molecules may be targeted simultaneously. On the other hand, this can be a disadvantage as well, due to the possibility of a greater degree of unwanted effects.

One requirement for specific inhibition of protein-protein interaction (PPI) and to limit potential adverse effects of drugs is to narrow down the targeting region. This will also increase alternative options for the choice of an application such as the utilization of peptide inhibitors or small-inhibitory molecules (discussed below).

Not all of the YxxP motifs in the Cas SD are phosphorylated by Src, or important for Crk, Src, and/or Nck binding (Kirsch *et al.* 2002; Shin *et al.* 2004) (summarized in Fig. 4), potentially suggesting that smaller fragments of the SD might be sufficient to mediate the inhibition of breast carcinoma progression. Importantly, the initial study describing the Src*/CasSD approach showed that the carboxy-terminal YxxP motifs six to fifteen of the p130^{Cas} SD were necessary for decoying/interacting with Crk (Kirsch *et al.* 2002). This correlated with the identified Crk-SH2 consensus sequence of YDxP (Birge *et al.* 1993; Songyang *et al.* 1993). In addition, the carboxy-terminal part of the p130^{Cas} SD is essential for p130^{Cas}-mediated cell migration (Shin *et al.* 2004). Furthermore, the majority of the 15 and 13 tyrosine motifs

within the p130^{Cas} and HEF1 SD, respectively, belong to two groups of sequences: YQxP and YDxP (Fig. 4B). PhosphoSitePlus™ (Hornbeck *et al.* 2004), a comprehensive resource of known phosphorylation sites, indicated that in both family members the amino-terminal motifs (primarily of the YQxP sequence) in the SD are rarely phosporylated, whereas the carboxy-terminal sites (primarily of the YDxP sequence) are significantly more often phosphorylated as measured by high-throughput mass spectometry screenings (Fig. 4B). In addition, these studies support the hypothesis that a drug based on the Src*/CasSD approach may block signaling mediated by both p130^{Cas} and HEF1 proteins. However, to date no studies addressing the influence of the Src*/CasSD chimera on HEF1 activity, and the importance of the carboxy-terminal part of the Cas SD for mediating the inhibition have been performed.

To test this hypothesis, experiments are currently ongoing in our laboratory using the Src*/CasSD approach to identify and define the smallest region of the SD that retains its inhibitory function in breast cancer *in vitro* and *in vivo*. Subsequently, the structure of this

constitutively phosphorylated peptide could be resolved by crystallization and *in silico* protein structure modeling, which may provide a starting point to design therapeutic agents for future testing.

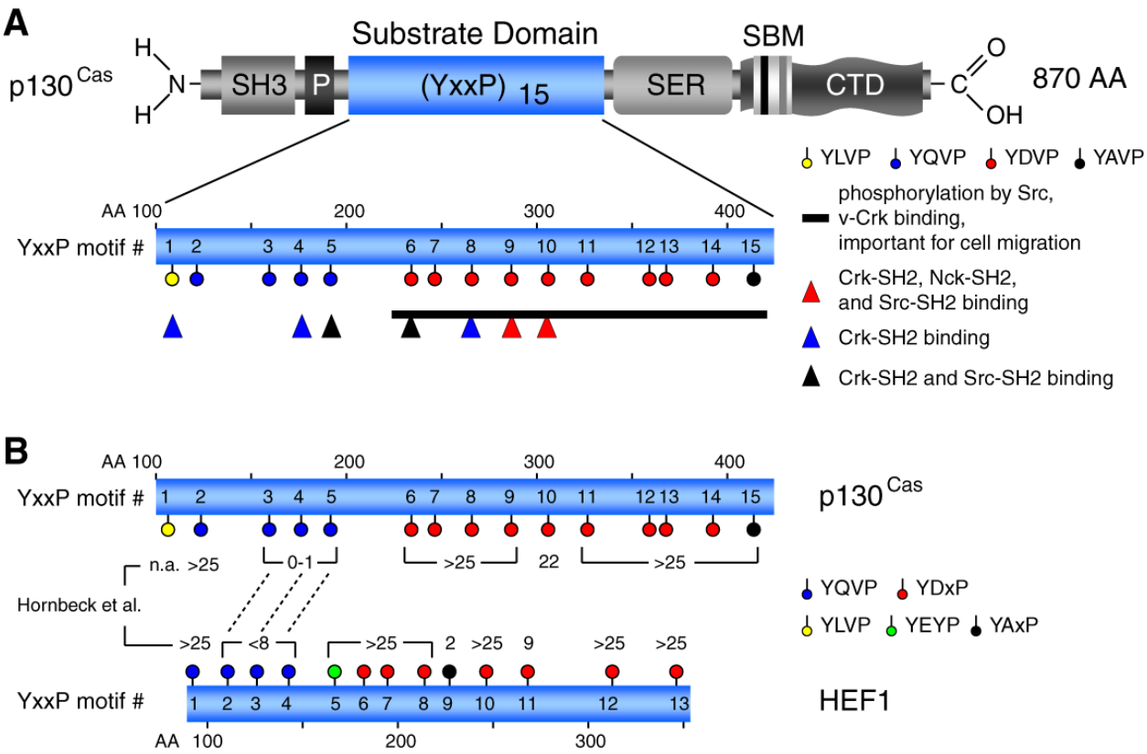

Fig. 4. Representation of the YxxP motifs in the p130Cas and HEF1 substrate domains. A, Interaction partners for p130Cas and a region important for cell migration are indicated. SH3, Src homology 3 domain. P, proline-rich region. SER, serine-rich domain. SBM, bi-partite Src-binding motif. CTD, carboxy-terminal domain. B, Comparison of the YxxP motifs in the SD of p130Cas and HEF1. The number of studies showing phosphorylation of certain motifs by high-throughput mass spectometry screening as curated at PhosphoSitePlus™ (Hornbeck *et al.* 2004) are indicated. n.a., not available.

4. Overview of potential approaches for targeting cytoplasmic adapter proteins

Over the past 15 years progress has been made in drug development and different novel approaches have become avaible to diversify the options of drug design and functional screening. For instance, humanized therapeutic antibodies, intrabodies, peptide inhibitors, peptidomimetics, or small-molecule inhibitors have been employed in cancer therapies and are undergoing clinical trials (Buchwald 2010; Leader *et al.* 2008; Lo *et al.* 2008).

Although therapeutic antibodies usually show a high specificity, they are in general not cell-permeable, thus excluding them as potential drug for targeting the intracellular Cas proteins. Intrabodies, antibodies designed to be expressed intracellularly, circumvent these obstacles and could possibly be directed to distinct subcellular locations (reviewed in Lo *et al.* 2008). It might be feasible to target a specific intracellular antigen e.g. in the nucleus, the endoplasmic

reticulum, mitochondria, or at the plasma membrane. This may represent an interesting approach to limit/focus the action of a drug to interfere with certain Cas protein functions.

Small molecules such as peptide inhibitors, peptidomimetics, or small-molecule inhibitors have recently become a focus for researchers to develop novel therapeutics. Great advances in the development of small molecules that modulate PPIs have been achieved (Arkin 2005; Arkin & Wells 2004; Wells & McClendon 2007). In the context of the Cas proteins that mediate their function as adapters by providing docking sites for multiple PPI, progress particularly in developing small-molecule inhibitors to block PPI is of major importance.

Peptide inhibitors represent potent therapeutic agents and over the past decade more than 50 peptides have been approved for the treatment of various diseases and several hundred are in preclinical development and clinical testing (Buchwald 2010). Advantages of peptide inhibitors are the high specificity, low toxicity, and low accumulation in tissues. However, peptide inhibitors exhibit a limited half-life in circulation, frequently possess restricted cell permeability, and are more expensive to produce than traditional small-molecule drugs. Often peptide inhibitors have become the starting point to subsequently develop PPI inhibitors (PPIIs) such as peptidomimetics (small protein-like chain based on a peptide with altered chemical structure designed to adjust the molecular properties to acheive increased stability or biological activity) or small-molecule inhibitors to circumvent these disadvantages.

Advances have been made in targeting PPI utilizing mimetic β-peptides (Kritzer *et al.* 2005), which consist of β amino acids that generally not appear in nature, thus increasing the resistance to proteolysis. Succesfully employed examples are β-peptides that target hDM2 (human double minutes-2, the human homologue of the murine p53 negative regulator MDM2) to prevent its interaction with the tumor suppressor p53 resulting in an upregulation of p53-dependent genes (Harker & Schepartz 2009; Kritzer *et al.* 2004). Importantly, subsequent structural modifications of these β-peptides enhanced their uptake in human coloncarcinoma cells.

In later stages of drug development, the goal would be to develop organic non-peptidic small-molecule inhibitors that are generally less expensive, cell-permeable and can be orally administered. Especially relevant to the CasSD approach, and proof of principle, is the progress made in the development of a small-inhibitory compound targeting the Grb2-HGFR (hepatocye growth factor receptor) interaction. The Grb2 antagonist C90, designed and validated to bind to the Grb2 SH2 domain thereby preventing specifically the association of Grb2 with phosphorylated tyrosine motifs within the HGFR (Atabey *et al.* 2001; Dharmawardana *et al.* 2006). The blockade of the Grb2 and HGFR interaction by the C90 small molecule resulted in the reduction of tumor metastases in two different mouse models (Giubellino *et al.* 2007) and inhibition of angiogenesis *in vivo* (Soriano *et al.* 2004). These studies elegantly demonstrate that small-molecule inhibitors possess the potential to block SH2 domain-mediated PPI with high specificity. Additional examples of small-inhibitory compounds with *in vivo* activity in cancer models include Nutlins (Vassilev 2007; Dickens *et al.* 2010), an inhibitor of MDM2, and the Bcl-2 blockers ABT-737 and ABT-263 (Vogler *et al.* 2009).

To summarize, progress has been made in developing different novel approaches in drug design for targeting cytoplasmic proteins. The successful application of small inhibitory compounds in preclinical and clinical trials shows their potential. Approaches employing small-molecule inhibitors may represent promising strategies to target specific regions and thus particular functions of Cas proteins.

5. Perspectives/Outlook on novel combinatorial adjuvant therapies for breast cancer

Increasingly, women are receiving adjuvant therapies in the form of chemotherapy, hormone- and/or radiation therapy (Yamashita 2008; Nicolini *et al.* 2006). Evidence is mounting that these therapies improve breast cancer survival. Adriamycin is one of the most frequently used agents for effective chemotherapeutic treatment of breast cancer (Gianni *et al.* 2008). Hormonal adjuvant therapy is a more targeted therapy with tamoxifen being a commonly used anti-estrogen for treatment of ERα positive breast cancers. Unfortunately, the use of these agents is limited by toxicity and the evolving resistance as seen for other chemotherapeutic drugs as well. This strongly indicates the need for the development of novel approaches to fight tumor cells and to prevent or reduce drug-resistance in breast cancer.

$p130^{Cas}$ was shown to be associated with tamoxifen (Dorssers *et al.* 2001) and adriamycin (Ta *et al.* 2008) resistance in human breast carcinomas *in vivo* and *in vitro*, respectively. Expression of Src*/CasSD in TAM-R cells re-sensitized TAM-R cells to tamoxifen (Soni *et al.* 2009) and RNAi-mediated depletion of $p130^{Cas}$ in breast cancer cells increased the efficiency of adriamycin treatment (Ta *et al.* 2008). Interestingly, expression of the Src*/CasSD in TAM-R cells attenuated signaling pathways which are also involved in $p130^{Cas}$-mediated adriamycin resistance, suggesting that the Src*/CasSD approach might increase the susceptibility to adriamycin as well. Implementation of the Src*/CasSD approach may potentially enhance the efficiency of other anti-estrogens, such as fulvestrant, or the dual tyrosine kinase inhibitor lapatinib (Tykerb), targeting EGFR and HER2/neu (Medina & Goodin 2008; Azim & Azim, Jr. 2008), as it results in an upregulation of ER alpha and HER2/neu in TAMR-R cells (Soni *et al.* 2009).

Data from these initial studies suggest that a therapeutic agent based on the Src*/CasSD approach may have the potential to open avenues for novel strategies for combinatorial adjuvant treatment of breast cancer in the future.

6. Conclusions/Summary

Although many studies indicate the critical involvement of the Cas family members in breast cancer progression, no therapeutic agents have been developed that target these proteins. Here we reviewed different approaches addressing this issue and presented in detail our SD decoy approach that represents an important novel tool to block endogenous Cas protein functions in mammary cancer. Ongoing studies with the aim to specify the region that is mediating these inhibitory effects may guide in the design of therapeutic agents in the future. As we discussed different future drug designs, small-molecule inhibitor approaches might be favourably suited for clinical applications to target Cas proteins, that may be extended to other PPI domains such as the SH3 and/or CTD, or SER domain.

7. Acknowledgments

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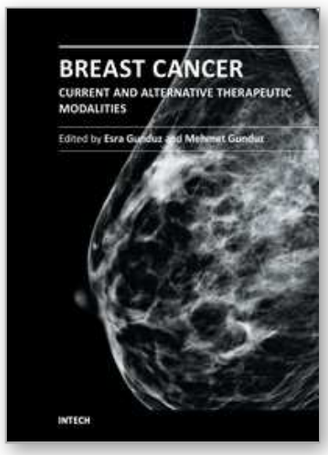

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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various therapeutic modalities from signaling pathways through various anti-tumor compounds as well as herbal medicine for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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