Functional Inactivation of CYLD Promotes the Metastatic Potential of Tumor Epidermal Cells

Josefa P. Alameda[1], M. Jesús Fernández-Aceñero[2], Rita M. Quintana[1], Angustias Page[1], Ángel Ramírez[1], Manuel Navarro[1] and M. Llanos Casanova[1]

CYLD is a tumor-suppressor gene mutated in the skin appendage tumors cylindromas, trichoepitheliomas, and spiradenomas. We have performed *in vivo* metastasis assays in nude mice and found that the loss of the deubiquitinase function of CYLD in squamous cell carcinoma (SCC) cells greatly enhances the lung metastatic capability of these cells. These metastases showed several characteristics that make them distinguishable from those carrying a functional CYLD, such as robust angiogenesis, increased expression of tumor malignancy markers of SCCs, and a decrease in the expression of the suppressor of metastasis Maspin. Restoration of Maspin expression in the epidermal SCC cells defective in CYLD deubiquitination function significantly reduces their ability to form metastases, thereby suggesting that the decrease in the levels of Maspin expression plays an important role in the acquisition of metastatic potential of these cells. In addition, we have characterized Maspin downregulation in cylindromas, trichoepitheliomas, and spiradenomas carrying functional inactivating mutations of *CYLD*, also providing an evidence of the correlation between impaired CYLD function and Maspin decreased expression *in vivo* in human tumors.

Journal of Investigative Dermatology (2013) **133,** 1870–1878; doi:10.1038/jid.2013.76; published online 21 March 2013

INTRODUCTION

CYLD is a tumor-suppressor gene mutated in hereditary syndromes that predisposes patients for the development of skin appendage tumors such as cylindromas, trichoepitheliomas, and spiradenomas (Gerretsen *et al.*, 1995; Bignell *et al.*, 2000; Lee *et al.*, 2005; Young *et al.*, 2006). Here we refer to all of these tumoral manifestations with the generic name of cylindromatosis. In addition to inherited tumors, these types of neoplasias are also found as sporadic tumors. CYLD functions as a deubiquitinase that removes the lysine-63 polyubiquitin chains from an array of target proteins involved in signal transduction and gene regulation (Brummelkamp *et al.*, 2003; Kovalenko *et al.*, 2003; Trompouki *et al.*, 2003). Most of the mutations within the *CYLD* locus are found in the catalytic residues of ubiquitin hydrolase (Massoumi and Paus, 2007; Saggar *et al.*, 2008).

We have recently reported on the role of CYLD in the homeostasis of the skin: it develops an important function in the maintenance of epidermal polarity, keratinocyte differentiation, and apoptosis (Alameda *et al.*, 2011a).

[1]*Department of Molecular Oncology, CIEMAT, Madrid, Spain and* [2]*Department of Pathology, Fundación Jiménez Díaz, Madrid, Spain*

Correspondence: M. Llanos Casanova, Department of Molecular Oncology, CIEMAT, Avenida Complutense 40, Madrid 28040, Spain. E-mail: llanos.casanova@ciemat.es

Abbreviations: NMSC, nonmelanoma skin cancer; SCC, squamous cell carcinoma

Received 5 December 2011; revised 8 January 2013; accepted 23 January 2013; accepted article preview online 20 February 2013; published online 21 March 2013

Nonmelanoma skin cancer (NMSC), i.e., basal cell carcinomas and cutaneous squamous cell carcinomas (SCCs), is the most common form of cancer in the Caucasian population (Christenson *et al.*, 2005). SCCs account for ~20% of all cutaneous malignancies, and may be very aggressive and invasive (Moller *et al.*, 1979). Among the factors considered to play a role as SCC promoters, we have recently shown that the diminished function of CYLD in tumor epidermal cells worsens the prognosis of malignant skin tumors through enhancement of the expression of angiogenic factors and increase in the nuclear localization of Bcl3, p52, and β-catenin (Alameda *et al.*, 2010, 2011a). In addition, we have shown that wild-type CYLD overexpression in human SCC cells reverts the malignant phenotype of the tumors (Alameda *et al.*, 2011a). It has also been reported that mice lacking *Cyld* are highly susceptible to development of chemically induced benign skin tumors (Massoumi *et al.*, 2006), and transgenic mice expressing a catalytically inactive mutant CYLD develop tumors of increased malignancy relative to nontransgenic mice (De Marval *et al.*, 2011). Here we have analyzed whether CYLD may also function as an inhibitor of metastasis of SCCs. To this aim, we have performed *in vivo* metastasis assays in nude mice and found that the expression in SCC cells of a mutant CYLD lacking the deubiquitinase function (and acting as a dominant negative interfering with the endogenous CYLD function) greatly enhances the lung metastatic capability of these cells. These metastases showed a strong angiogenesis and the induction of markers of high aggressiveness of SCCs such as Snail and keratins K8 and K13. In addition, they also exhibit a

downregulation of the inhibitor of metastasis Maspin, and we provide evidences suggesting that this decreased expression of Maspin is, at least in part, responsible for the enhancement of the metastatic capacity of the SCC cells lacking the deubiquitinase function of CYLD.

Maspin is a protein of the Serpin family that acts as a tumor suppressor inhibiting cellular motility, invasiveness, and angiogenesis, and providing sensitivity to apoptosis in tumor cells (Bailey *et al.*, 2006; Khalkhali-Ellis, 2006). Maspin expression predicts a better prognosis in different types of cancers: breast, prostate, colon, oral squamous cell carcinoma, lung, larynx, malignant melanoma, and ovarian cancer (Shi *et al.*, 2001; Futscher *et al.*, 2004). Maspin also acts as suppressor of metastasis in different types of cancer such as prostate, liver, and breast (Luo *et al.*, 2007; Stark *et al.*, 2010). Little is known about the expression of Maspin in skin and skin cancer. We have recently found that the diminished expression of Maspin in mouse skin tumors is a potential cause for NMSC progression (Alameda *et al.*, 2011b).

Our present data show an inverse relationship between the grade of malignancy of tumor epidermal cells and the level of expression of Maspin. We have also found a correlation between forced CYLD functional inactivation in epidermal SCC cells and a decreased Maspin expression. This correlation is also found *in vivo* in cylindromatosis tumors carrying a mutated *CYLD* gene. In addition, we have found a physical interaction between CYLD and Maspin.

RESULTS

The inhibition of CYLD function in tumor epidermal cells enhances its capacity to generate lung metastases

Previously, we introduced the mutant CYLD$^{C/S}$ complementary DNA in the PDVC57 cell line. This mutant carries the 601$^{C/S}$ point mutation in the cysteine box of the deubiquitinase domain, resulting in a catalitically inactive protein that is able to compete with the endogenous CYLD. We found that the expression of the CYLD$^{C/S}$ mutant makes the squamous cancer PDVC57 cells (SCC cells) very aggressive, conferring them properties such as capacity of growing in suspension and increased angiogenesis that finally leads to a rapid tumor development in carcinogenesis assays (Alameda *et al.*, 2010, 2011a). Now we have performed *in vivo* metastasis assays to ascertain whether CYLD has a role in the metastatic behavior of SCC epidermal cells. We injected the C57-Control cell (expressing the empty vector) and C57-CYLD$^{C/S}$ cells (expressing the CYLD$^{C/S}$ transgene) in the tail vein of immunodeficient mice. Soon after injection (18 days), 33% of the C57-CYLD$^{C/S}$ -injected mice showed cachexia, being extremely thin and displaying evident breathing difficulties. The autopsy of the animals showed enlarged lungs of brownish-red color, with a dried appearance and hard consistency (Figure 1a); moreover, lungs injected with the C57-CYLD$^{C/S}$ cells appeared full of metastases that covered almost all its surface (Figure 1b). The appearance of these lungs was in sharp contrast with the whitish, spongy, and moist appearance of the lungs injected with the C57-Control cells (Figure 1a).

The histological analysis of the lungs of the injected mice showed that the C57-Control cells also led to the development

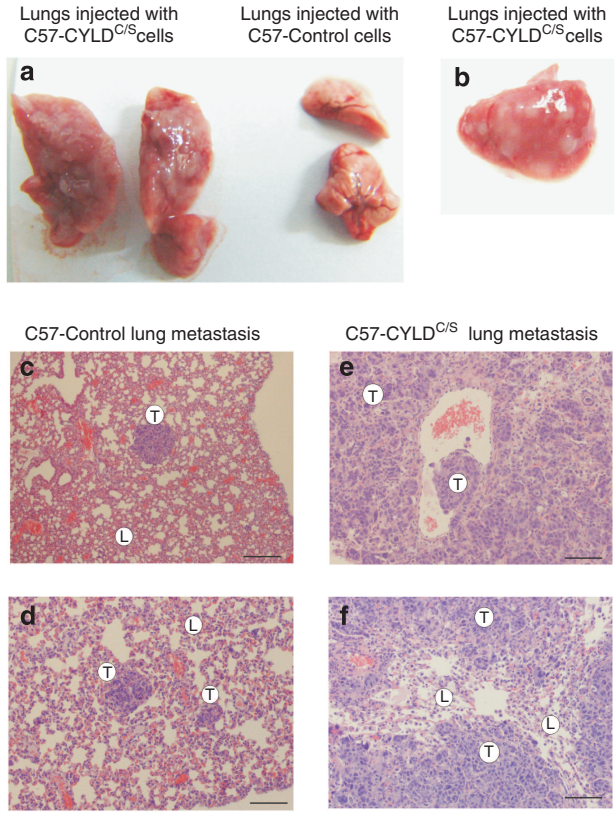

Figure 1. Histological characterization of lungs injected with C57-CYLD$^{C/S}$ and C57-Control cells. (**a**) Representative image of the macroscopic appearance of lungs injected with C57-CYLD$^{C/S}$ or C57-Control cells collected 18 days after injection of the corresponding C57 cells. Note that each lung injected with the C57-CYLD$^{C/S}$ cells doubles the size of the lungs injected with the C57-Control cells. (**b**) Detail of the abundant metastasis (white nodules) present in the lungs injected with the C57-CYLD$^{C/S}$ cells. (**c–f**) Hematoxylin–eosin staining of sections corresponding to (**c**, **d**) C57-Control metastasis and (**e**, **f**) C57-CYLD$^{C/S}$ metastasis. (**e**) Note the C57-CYLD$^{C/S}$ tumor cells breaking through a blood vessel wall. Observe the scarce normal lung tissue remaining in the lungs injected with the C57-CYLD$^{C/S}$ cells. L, lung tissue; T, tumor mass. Bars = 300 μm.

of metastasis (C57-Control-metastases). However, the area occupied by the C57-CYLD$^{C/S}$-metastases (metastasis originated by the injection of the C57-CYLD$^{C/S}$ cells) was nearly 90% versus 10% of the area occupied by the C57-Control-metastases (Supplementary Table S1 online). The C57-CYLD$^{C/S}$ cells invading the lungs formed pleomorphic, poorly differentiated SCCs (Figure 1e and f). In contrast, the C57-Control cells formed moderate to well-differentiated SCCs (Figure 1c and d). To further confirm that the metastatic masses observed in the lungs were epidermal cells, we performed immunostaining with keratin K5, characteristic of basal keratinocytes of stratified epithelia such as skin and also expressed in SCCs, but not present in the lungs. C57-CYLD$^{C/S}$-metastasis showed extensive staining of K5 (Figure 2c), similar to that found in C57-SCCs (Figure 2a), demonstrating a massive colonization of the lung tissue by epidermal-origin C57-CYLD$^{C/S}$ cells. In contrast, C57-Control-metastasis only showed small colonies of epidermal cells positive for K5 (Figure 2e). K6, a keratin expressed by epidermal cells subjected to stress or proliferative

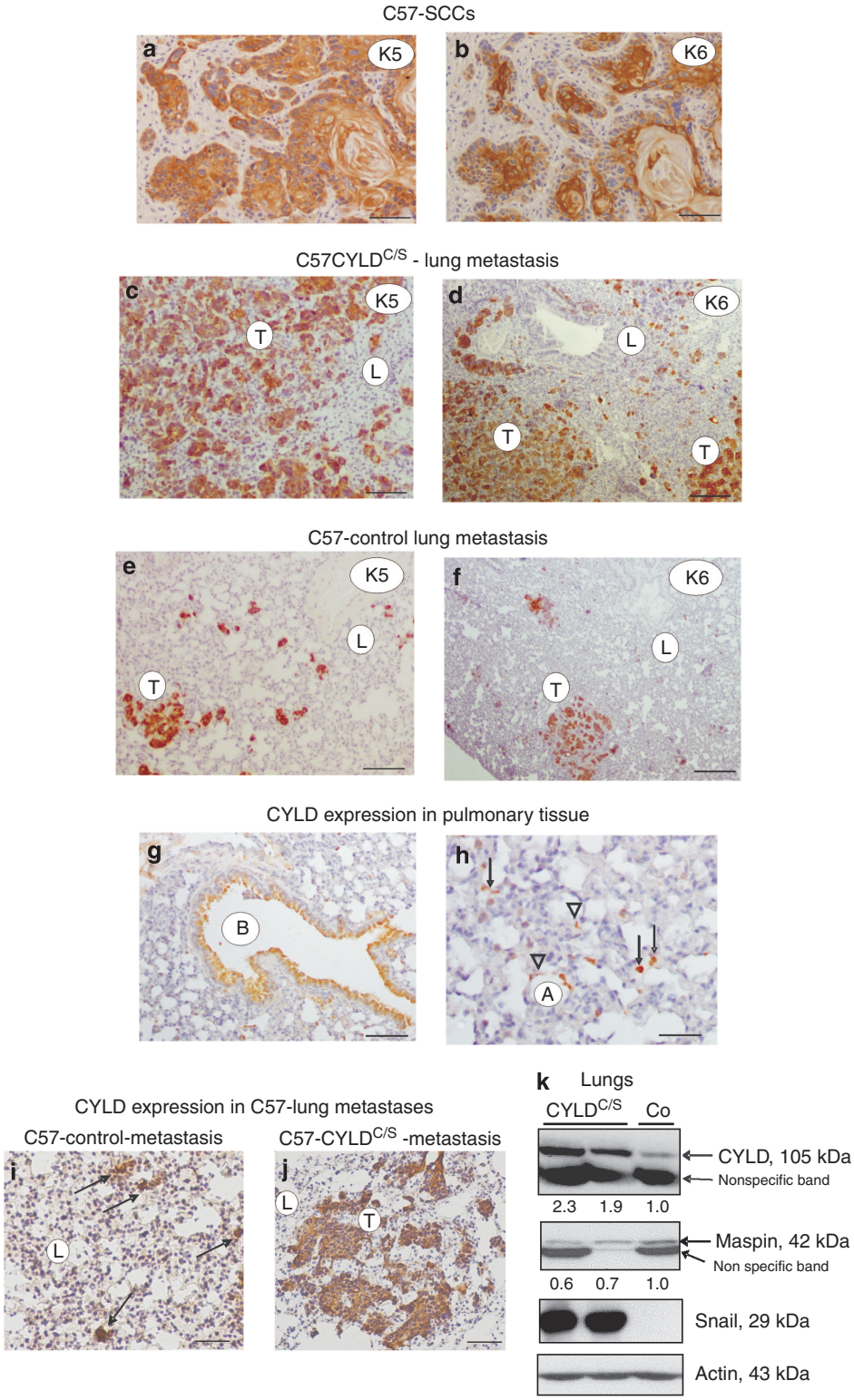

Figure 2. CYLD expression in normal lungs and lung metastasis; characterization of lung metastasis. (**a**, **b**) Keratin 5 and 6 (K5 and K6) staining of the squamous cell carcinomas (SCCs) obtained by subcutaneous inoculation of C57 cells in nude mice. (**c–f**) K5 and K6 staining showing the epidermal nature of the metastasis. (**g**, **h**) CYLD expression in normal lungs. It is detected in the apical epithelium of the bronchioles (B), pneumocytes type I of the alveoli (A, arrowheads), and in macrophages (arrows). (**i, j**) CYLD expression in the (**i**) Control-metastasis and (**j**) CYLD$^{C/S}$–metastasis. (**k**) Immunoblot comparing CYLD, Snail, and Maspin expression in lungs containing both C57-Control and CYLD$^{C/S}$-metastases (collected 26 days after injection). L, lung tissue; T, tumor mass. Bars: (**a–f**) = 350 μm; (**g**, **h**) = 200 μm; (**i**, **j**) = 300 μm.

conditions and skin SCCs (Figure 2b), was also detected in the metastases invading the lungs (Figures 2d and f), further confirming the epidermal origin of the tumoral masses.

We next analyzed by immunohistochemistry the expression of CYLD in normal lungs as well as in C57-Control-metastases and C57-CYLD$^{C/S}$-metastases. In the normal lungs, we found that CYLD was expressed in the epithelium of the bronchioles (in particular in the apical region; Figure 2g) as well as in the type I pneumocytes of the alveoli and in macrophages (Figure 2h). This pattern of expression was also detected in lungs carrying C57-Control-metastasis (Supplementary Figure 1a online); however, endogenous CYLD was weakly detected in lungs carrying C57-CYLD$^{C/S}$-metastasis (Supplementary Figure1b online). We found elevated expression of CYLD in the epidermal cancer cells forming the C57-CYLD$^{C/S}$-metastasis (Figure 2j), whereas in the C57-Control-metastasis only small aggregates of tumor cells positive for CYLD staining were found (Figure 2i) Figure 2k shows a western blot also indicating the increased CYLD expression in the C57-CYLD$^{C/S}$-metastasis.

In addition to lung, other tissues were analyzed (kidney and brain) and no metastases were found (data not shown).

C57-CYLD$^{C/S}$-metastases show markers distinctive of highly aggressive epidermal tumors such as increased angiogenesis, induction of Snail and keratins K8/K13, and decreased expression of Maspin

We analyzed the expression of markers of malignancy of SCCs such as keratins K8 and K13 in the lung metastases. K8 is characteristic of simple epithelia (Moll *et al.*, 1982); although it is not expressed in epidermis, it is frequently detected in skin tumors, with this aberrant expression being considered a marker of malignancy (Casanova *et al.*, 2004). In lung tissue, K8 was detected in the respiratory epithelium that lines the bronchi, bronchioles, and alveoli, as expected (Casanova *et al.*, 1995). K8 was not detected in the C57-Control epidermal cells invading the lungs (Figure 3a); however, it was strongly expressed in the tumor cells of the C57-CYLD$^{C/S}$-metastasis, indicating its high grade of malignancy (Figure 3b). K13 are a keratin characteristic of internal stratified squamous epithelia that is aberrantly expressed in skin tumors and are also considered a marker of tumor progression (Winter *et al.*, 1990; Moreno-Maldonado *et al.*, 2008). We found K13-positive cells in the C57-CYLD$^{C/S}$-tumoral masses invading the lungs (Figure 3d), whereas it was not detected in the C57-Control-metastasis (Figure 3c). We also found that C57-CYLD$^{C/S}$-metastasis were highly proliferative as indicated by the elevated number of positive cells for Ki67 (Figure 3f) compared with that detected in the C57-Control-metastasis (Figure 3e).

Another factor that contributes to increase the aggressiveness of SCCs is the presence of a strong vascularization (Bolontrade *et al.*, 1998). We have analyzed by smooth muscle actin immunostaining the pattern of blood vessels in both C57-Control-metastasis and CYLD$^{C/S}$-metastasis and found that C57-CYLD$^{C/S}$-metastases were highly vascularized (Figure 3h), whereas we hardly detected smooth muscle actin–positive staining in the C57-Control-metastasis (Figure 3g).

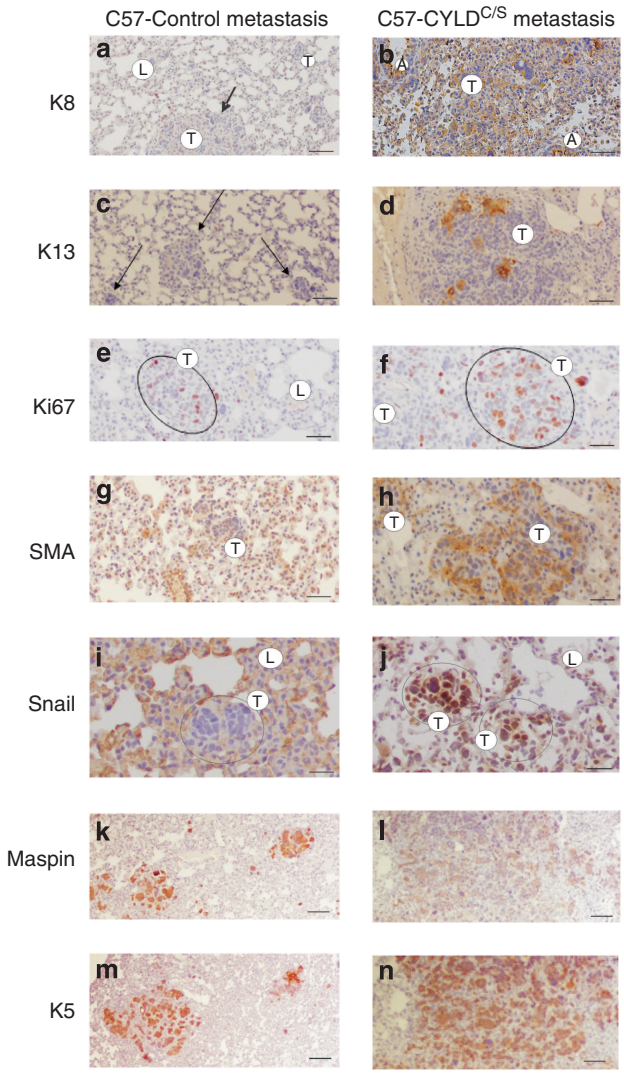

Figure 3. Analysis of markers of malignancy in C57-Control and C57-CYLD$^{C/S}$metastases. (**a**, **b**) Keratin 8 (K8) immunostaining. (**c**, **d**) Keratin 13 (K13) staining. (**e**, **f**) Ki67 staining showing the proliferation rate of the C57-control and C57-CYLD$^{C/S}$-metastasis. (**g**, **h**) Smooth muscle actin (SMA) staining of the blood vessels of C57-Control-metastasis and CYLD$^{C/S}$-metastasis is used as indicative of the tumor angiogenesis. Note the strong vascularization of the metastasis expressing the mutant CYLD$^{C/S}$. Snail expression was detected in the (**j**) C57-CYLD$^{C/S}$-metastasis but not in the (**i**) C57-Control-metastasis. (**l**) Observe the diminished expression of Maspin in the CYLD$^{C/S}$ squamous cell carcinomas (SCCs). (**m**, **n**) K5 staining shows the extension of the C57-Control-metastasis and CYLD$^{C/S}$-metastasis. A, alveoli; L, lung tissue; T, tumor mass. Bars: (**a**–**d**) = 350 μm; (**e**, **f**) = 250 μm; (**g**–**j**) = 200 μm; (**k**–**n**) = 300 μm.

This result is in agreement with our previous finding showing an enhanced angiogenesis in the skin tumors obtained by subcutaneous inoculation of both the murine C57 and human A431 SCC cells expressing the CYLD$^{C/S}$ mutant (Alameda *et al.*, 2010, 2011a).

As it has been described that Snail develops an important role in mouse skin tumor progression and invasion (Peinado *et al.*, 2004; Roy *et al.*, 2004; Barrallo-Gimeno and Nieto, 2005), we analyzed by both immunohistochemistry and western blot whether Snail was expressed in the metastasis.

We found elevated expression of Snail in the C57-CYLD$^{C/S}$-metastasis, whereas it was not detected in the C57-Control-metastasis, therefore suggesting a more aggressive phenotype of the C57-CYLD$^{C/S}$-metastasis (Figures 2k and 3i and j).

We have recently found a correlation between decreased levels of Maspin and increased malignancy of epidermal tumors in mice (Alameda *et al.*, 2011a). We then checked the expression of Maspin in the metastasis and found that it was strongly expressed in the C57-Control-metastasis (Figure 3k), almost in the whole tumor mass (denoted by the K5 expression area, Figure 3m); in contrast, it showed a weak expression in the tumor cells of C57-CYLD$^{C/S}$-metastasis (Figure 3l), in spite of the large size of the SCCs (indicated by K5 expression, Figure 3n). Western blot analysis also showed decreased levels of Maspin in C57-CYLD$^{C/S}$-metastasis (Figure 2k).

CYLD lack of function directly correlates with Maspin decreased expression in epidermal SCC cells

The above analysis shows striking differences between C57-Control and C57-CYLD$^{C/S}$ metastasis for expression of two important proteins for metastasis progression, i.e., Snail and Maspin. We have then examined whether the changes in the expression of both Snail and Maspin have previously taken place in the C57-CYLD$^{C/S}$ cells growing in monolayer cultures. qPCR analysis showed a 4-fold increase in the expression of Snail in the C57-CYLD$^{C/S}$ cells with respect to that in the C57-Control cells (Figure 4a). However, we could not detect Snail protein by western blot in C57-Control cells nor in C57-CYLD$^{C/S}$ cells (Figure 4b) growing in monolayer cultures. The analysis of Maspin expression by qPCR showed that levels were not significantly different between C57-control or C57-CYLD$^{C/S}$ cells (data not shown). However, the analysis of Maspin protein showed that the C57-CYLD$^{C/S}$ cells express lower levels of Maspin than the C57-Control cells (Figure 4c). To discard a cell type–specific effect, we studied whether the mutant CYLD isoform affected the expression of Maspin in two other cell lines: the A431 SCC cells and the murine PB cells (these cell lines were transfected with the CYLD$^{C/S}$ construct and A431-CYLD$^{C/S}$ and PB-CYLD$^{C/S}$ clones isolated). We found that effectively, both A431-CYLD$^{C/S}$ and PB-CYLD$^{C/S}$ cells exhibited lower levels of Maspin expression than their respective controls (A431-Control and PB-Control cells; Figures 4d and e). These results indicate a correlation between the loss of the catalytic function of CYLD and the diminished expression of Maspin in tumor SCC cells. We then analyzed whether the overexpression of exogenous wild-type CYLD in SCC cells has the opposite effect. For this purpose, we studied the expression of Maspin in A431-CYLD^{WT} cells (A431 cells overexpressing the CYLD wild-type complementary DNA) (described in Alameda *et al.*, 2011a) and observed higher levels of Maspin expression in these cells as compared with A431-Control cells (Figure 4d). Therefore, our results show that the impairment of the deubiquitination function of CYLD in SCC cells as a result of CYLD$^{C/S}$ expression is accompanied by a decrease in the expression of Maspin protein in these tumor cells and, conversely, the exogenously increased CYLD wild-type expression correlates with an increased expression of Maspin. According to these results, we have performed immunoprecipitation experiments that suggest a possible physical interaction between CYLD and Maspin (Supplementary Figure S2 online).

In order to analyze whether the reduction in the expression of Maspin in the C57-CYLD$^{C/S}$ cells could be a reason for their highly malignancy, we have transfected them with a complementary DNA of Maspin. Double transfectans, C57-CYLD$^{C/S}$-Maspin cells, were selected (Figure 4g). A reduction in the proliferation rate of the double transfectans was observed in monolayer cultures of C57-CYLD$^{C/S}$–Maspin cells compared with both C57-Control and C57-CYLD$^{C/S}$ cells (Figure 4h). The metastasis assays in nude mice showed that whereas the C57-CYLD$^{C/S}$ cells developed abundant foci of metastasis by day 5 after injection (Figure 4j and Supplementary Table S2 online), the C57-CYLD$^{C/S}$–Maspin cells only originate few small metastases per lung (Figure 4k, Supplementary Table S2 online). At this time, C57-Control cells also develop metastases, although less numerous and smaller in size than the C57-CYLD$^{C/S}$ cells (Figure 4i, Supplementary Table S2 online). K5 staining confirmed the epidermal nature of the metastases (Figure 4i–k). We have also detected a diminished vascularization of the C57-CYLD$^{C/S}$–Maspin metastasis with respect to that of the C57-CYLD$^{C/S}$ metastasis (Supplementary Figure S3 online).

An inverse relationship between levels of Maspin expression and the grade of malignancy of epidermal cells is detected in different cell lines

As the expression of Maspin in epidermal cells has not been explored yet, we studied whether a relationship exists between Maspin expression and the grade of malignancy of epidermal tumor cells. We found that both murine (MCA3D) and human (HaCaT) nontumoral cells expressed higher levels of Maspin than the tumoral cells. The tumor SCC13 human cells, which are less aggressive than the A431 cells, express higher levels of Maspin (Figure 4f). We also found that C57 murine tumor cells expressed lower levels of Maspin than normal mouse skin and MCA3D cells; and the very aggressive HaCa4 SCC cells express low levels of Maspin (Figure 4f). Overall, our results establish a decreased expression of Maspin with increasing tumor epidermal cell malignancy, suggesting that the low levels of Maspin expression results in a more invasive phenotype.

Maspin expression is decreased in human cylindromas, trichoepitheliomas, and spiradenomas defective in CYLD function

In order to analyze whether there is a correlation *in vivo* between CYLD lack of function and a decrease in Maspin expression, we studied the expression of Maspin in cylindromas, spiradenomas, and trichoepitheliomas (lacking CYLD deubiquitinase function). Skin from healthy donors showed that Maspin was mainly expressed in the suprabasal, differentiated layers of the epidermis, and in the inner root sheath of hair follicles (Figure 5a; compare with the K14 basal staining and the suprabasal K10 expression, Figure 5b and c). Staining of the cylindromatosis tumors showed that Maspin was

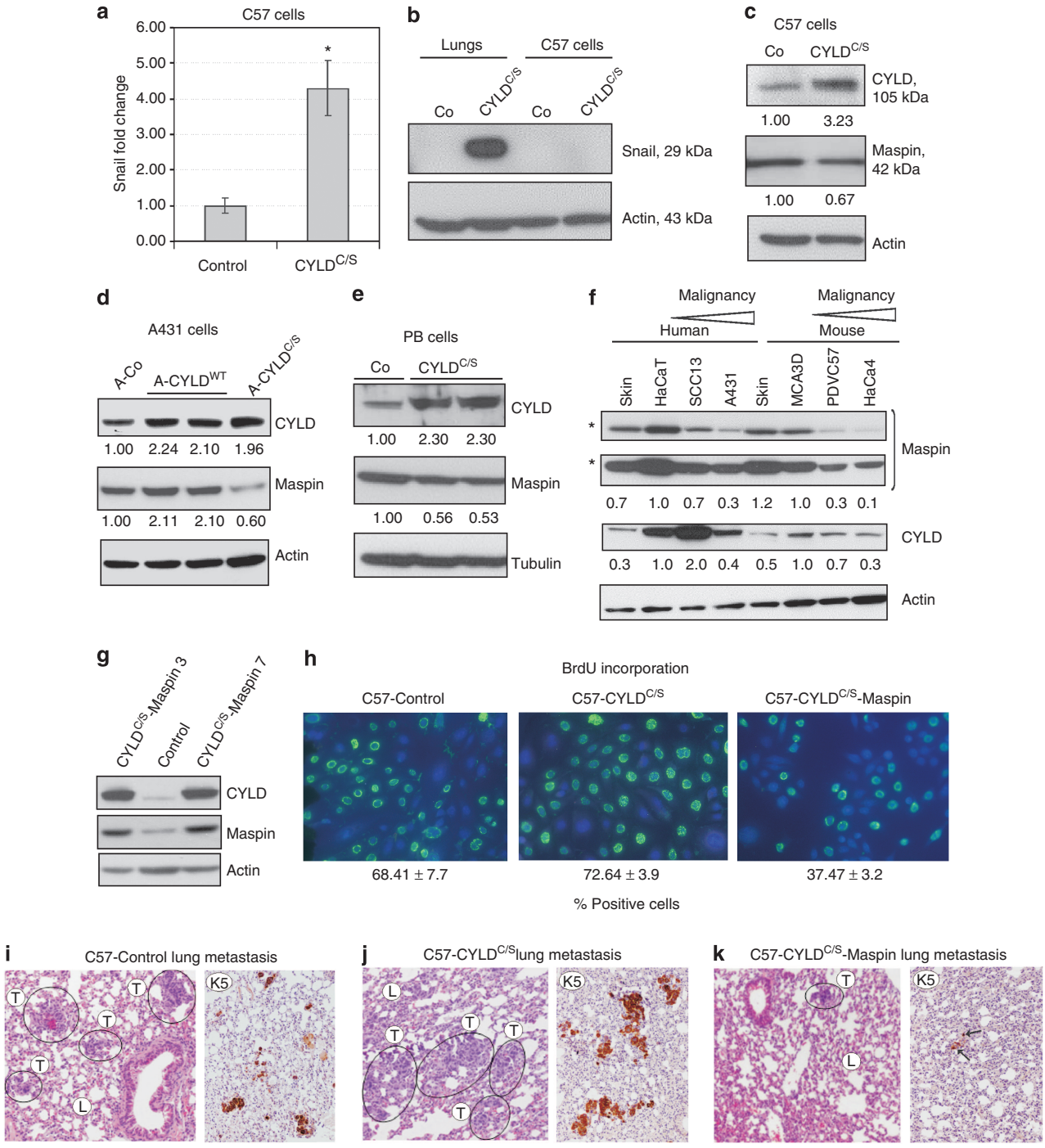

Figure 4. Snail and Maspin expression in human and murine cells. (**a**) qPCR showing significant increase in Snail expression in C57-CYLD$^{C/S}$ cells (*$P<0.05$). (**b**) Snail protein is not detected in the C57-Control not in the C57-CYLD$^{C/S}$ cells. Lungs containing C57-CYLD$^{C/S}$-metastasis (positive for Snail expression) and C57-Control-metastasis (negative for Snail protein) were used as controls. (**c–e**) Downregulation of Maspin in three types of CYLD$^{C/S}$ cells. (**d**) A431-CYLD^{WT} cells show enhanced Maspin expression. (**f**) Observe a correlation between decreased Maspin expression and increasing malignancy. CYLD expression levels are shown. Asterisks indicate different exposition times. (**g**) Increased Maspin expression in the C57-CYLD$^{C/S}$–Maspin cells (clones 3 and 7). (**h**) C57-CYLD$^{C/S}$–Maspin cells show decreased proliferation rate (36-h culture). (**i–k**) Hematoxylin/eosin and keratin 5 (K5) staining showing the reduced number and size of metastasis in the C57-CYLD$^{C/S}$–Maspin lungs. Co, control; L, lung tissue; T, tumor mass.

strongly expressed in the adjacent healthy epidermis, whereas it was weakly expressed in the tumoral cells of the three types of neoplasias (Figure 5g–l). In cylindromas and trichoepitheliomas, Maspin was only located in the inner parts of the tumor islands that are the more differentiated regions (Figure 5g, j, h, and k). In spiradenomas, scarce Maspin expression was detected in sporadic cells of the tumor compared with the high and continuous expression seen in

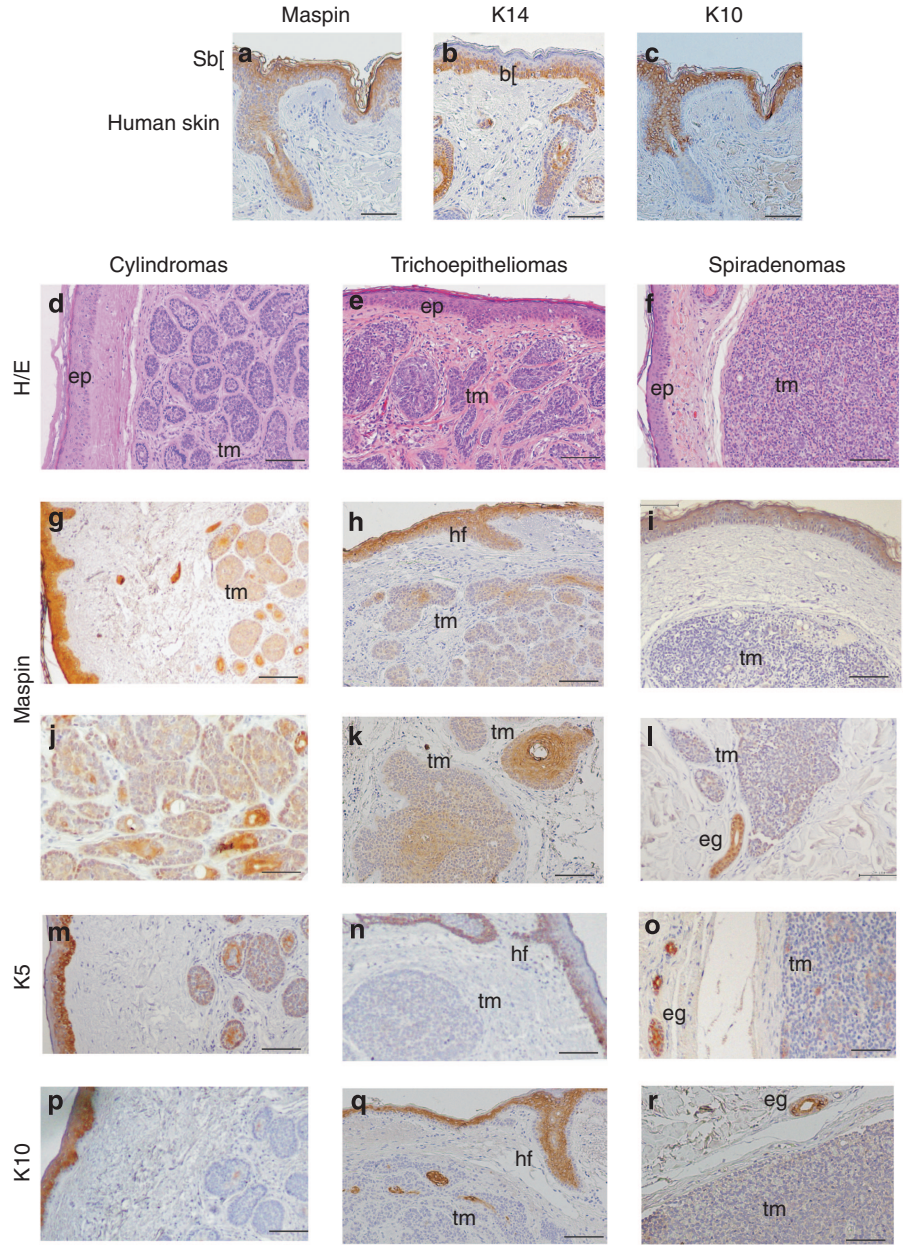

Figure 5. Maspin, keratin 5 (K5), and keratin 10 (K10) expression in human skin, cylindromas, trichoepitheliomas, and spiradenomas. (**a–c**) Expression of Maspin in the suprabasal layers of the skin and the outer root sheath of the hair follicles. K14 and K10 staining mark the basal and suprabasal cell layers of the epidermis, respectively. (**d–f**) Hematoxylin/eosin (H/E) staining. (**g–l**) Maspin expression in the three types of tumors. (**m–o**) K5 immunostaining. (**p–r**) K10 expression. b, basal; eg, eccrine gland; ep, epidermis; hf, hair follicle; Sb, suprabasal; tm, tumor mass. Observe the decreased expression of K5, K10, and Maspin in the tumor masses compared with the elevated expression in epidermis, hair follicles, and eccrine glands. An average of five samples of each case was analyzed. Bars: (**a–c**) = 500 μm; (**d–i**) = 400 μm; (**j–r**) = 350 μm.

the epidermis and the eccrine glands (Figure 5i and l). We observed that keratin K5 and K10 expression was also down-regulated in most of the tumor islets of the three types of familial cylindromatosis tumors (Figure 5m–r). These results show that, as occurs in CYLD$^{C/S}$ tumor cells, where the lack of the deubiquitination function of CYLD was accompanied by diminished expression of Maspin, in tumor cells of cylindromatosis patients carrying a functionally similar inactivating mutation in the *CYLD* gene (Rajan *et al.*, 2009), a decreased expression of Maspin was also observed.

DISCUSSION

Here we describe that in *in vivo* metastasis experiments in nude mice, the epidermal C57-CYLD$^{C/S}$ cells lacking the deubiquitinase function of CYLD leads to very aggressive metastasis in lung. Previously, we had found that lack of CYLD function in epidermal cells leads to an increase in several angiogenic factors such as vascular endothelial growth factor; it also makes the cells resistant to apoptosis, increases their proliferation, and induces the delocalization of adhesion proteins (E-cadherin and β-catenin) (Alameda *et al.*, 2010,

2011a). Altogether, the combination of all these alterations could be responsible for the great increase in the metastatic potential of the C57-CYLD$^{C/S}$ cells. In addition, we have now found decreased expression of Maspin in the C57-CYLD$^{C/S}$ cells, which appears as an important factor for the increased malignancy of the C57-CYLD$^{C/S}$ cells, as restoration of Maspin expression in C57-CYLD$^{C/S}$ cells results in reduction in the number and extension of metastases in the lung.

We have found evidences indicating a direct correlation between the lack of CYLD function as a consequence of the expression of the mutant CYLD$^{C/S}$ in SCC cells and the diminished expression of Maspin (Figure 4c–e). Moreover, our results also show that the exogenous overexpression of CYLD^{WT} in SCC cells correlates with increased levels of Maspin (Figure 4d). Remarkably, we have found loss of Maspin expression in the CYLD-deficient tumors (cylindromas, trichoepitheliomas, and spiradenomas) compared with the expression in normal human skin, indicating a correlation *in vivo* between the deficient CYLD function and the decrease in Maspin expression as well. The fact that most of the hereditary cylindromatosis patients do not develop metastasis may be likely because of the lack of additional mutations (besides CYLD mutation) in this kind of tumors.

It had been reported that Maspin is an important inhibitor of human tumor progression and metastasis in different types of cancers such as breast, liver, and prostate carcinomas (Luo *et al.*, 2007; Stark *et al.*, 2010). However, until now, there is little information about the relation of Maspin and skin cancer. Recently, we have reported a possible relationship between Maspin downregulation and NMSC progression *in vivo* in mouse skin carcinogenesis assays (Alameda *et al.*, 2011b). Here we have found a correlation between decreased expression of Maspin and increased malignancy of mouse and human tumor epidermal cells. These results suggest that the levels of Maspin expression could be an important predictive tool in the course of the NMSC progression and metastasis.

We have found that a relationship exists between lack of CYLD function in epidermal SCCs and increased lung metastatic capacity of the tumor cells. Our results contribute to the understanding of the molecular biology of SCC epidermal metastasis, assigning a role for CYLD as suppressor of lung metastasis in addition to its implication in the familial cylindromatosis disease. Our data suggest that the reduction in the levels of Maspin expression in the C57-CYLD$^{C/S}$ cells—along with the other changes previously described to occur in the C57 cells as a consequence of CYLD$^{C/S}$ expression—plays an important role in the acquisition of high metastatic potential of these cells.

MATERIALS AND METHODS

Cell lines and culture conditions

C57, A431, and PB cells were cultured in DMEM supplemented with 10% fetal calf serum. Transfected cells were grown in the presence of 0.4 mg of G418 per ml for single transfection or 0.4 mg of G418 per ml plus 0.1 mg of Hygromciyn B per ml for double transfection (C57-CYLD$^{C/S}$–Maspin cells). C57-Control, C57-CYLD$^{C/S}$, A431-Control, A431-CYLD$^{C/S}$, and A431-CYLD^{WT} cells have been already described (Alameda *et al.*, 2010, 2011a).

DNA constructs

Constructs employed have been previously described (Alameda *et al.*, 2010, 2011a). Maspin complementary DNA of rat origin (Tokuyama *et al.*, 2007) was subcloned into the pcDNA3-hygromycin (Invitrogen, Carlsbad, CA).

Metastasis assays

First time, 5×10^5 C57-Control and C57-CYLD$^{C/S}$ cells were resuspended in 100 μl of phosphate-buffered saline and injected intravenously into the tail vein. A total of 10 nude mice (Hsd-Athymic Nude, Harlan Europe, Barcelona, Spain) were injected with cells of each phenotype. For metastasis experiments with C57-Control, C57-CYLD$^{C/S}$, and C57-CYLD$^{C/S}$–Maspin cells (Figure 4i–k), 5×10^5 cells of each genotype were resuspended in 100 μl of phosphate-buffered saline and injected intravenously into the eye vein. Then, 7 nude mice were injected with cells of each phenotype and samples were recovered 5 and 10 days after injection. All experimental procedures were performed according to European and Spanish laws.

Histology and immunohistochemistry

Formalin-fixed lungs were stained with hematoxylin and eosin for histopathological evaluation and immunohistochemistry was performed with the following antibodies: K5, K6, K10 (Covance, San Diego, CA); CYLD (Sigma-Aldrich, St Louis, MO); K8, smooth muscle actin (Sigma-Aldrich); K13, Snail (Abcam, Cambridge, UK); Ki67, K14 (NeoMarkers, Fremont, CA); and Maspin (Santa Cruz Biotechnology, Heidelberg, Germany). Stainings with CYLD and Snail antibodies were performed in cryosections fixed in methanol/acetone.

Western blot analysis

Antibodies used were: Actin, Maspin, Snail, rabbit IgG (Santa Cruz Biotechnology, Europe); CYLD (SAB4200060, SAB4200061); and tubulin (Sigma-Aldrich). A total of 40–50 μg of total lysate was used. Total protein extracts (300 μg) were immunoprecipitated with an antibody specific for CYLD (SAB4200060 or SAB4200061) or Maspin. These three antibodies were produced in rabbit. Proteins were resolved on an SDS gel; probed with anti-Maspin and CYLD SAB4200060 or CYLD (sc-25779, Santa Cruz Biotechnology) antibodies. As control of specificity, samples (300 μg) were immunoprecipitated with rabbit IgG.

Quantitative PCR

RNA was extracted from cells using trizol followed by purification by RNeasy columns. RNA was reverse transcribed using the ABI High-Capacity Kit (ABI, Foster City, CA). Real-Time PCR was performed using predesigned Taqman assays in an ABI 7500Fast instrument, using the conditions indicated by ABI.

Human tissue samples

For formalin-fixed, paraffin-embedded skin and tumor samples, archival paraffin blocks were obtained from the biobank of the Fundación Jiménez Díaz (Madrid, Spain). All experimentation was approved by the Research Committee of the Hospital Fundación Jiménez Díaz, and this study has been designed and performed following Declaration of Helsinki Principles for human research in their revised form.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Dr R Bernards for providing the CYLD$^{C/S}$ cDNA; Federico Sánchez-Sierra for invaluable assistance in the histological techniques; Cristina Mera for immunohistochemical support; CIEMAT Animal Unit for care of the mice; and Dr Satomura and Dr Asamoto for providing the Maspin cDNA. This work was funded by grants from the *Ministerio de Ciencia e Innovación* PI10/01480 to MLC, and SAF2010-22156 to AR.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Alameda JP, Fernandez-Acenero MJ, Moreno-Maldonado R *et al.* (2011a) CYLD regulates keratinocyte differentiation and skin cancer progression in humans. *Cell Death Dis* 2:e208

Alameda JP, Moreno-Maldonado R, Fernandez-Acenero MJ *et al.* (2011b) Increased IKKalpha expression in the basal layer of the epidermis of transgenic mice enhances the malignant potential of skin tumors. *PLoS One* 6:e21984

Alameda JP, Moreno-Maldonado R, Navarro M *et al.* (2010) An inactivating CYLD mutation promotes skin tumor progression by conferring enhanced proliferative, survival and angiogenic properties to epidermal cancer cells. *Oncogene* 29:6522–32

Bailey CM, Khalkhali-Ellis Z, Seftor EA *et al.* (2006) Biological functions of maspin. *J Cell Physiol* 209:617–24

Barrallo-Gimeno A, Nieto MA (2005) The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* 132:3151–61

Bignell GR, Warren W, Seal S *et al.* (2000) Identification of the familial cylindromatosis tumour-suppressor gene. *Nat Genet* 25:160–5

Bolontrade MF, Stern MC, Binder RL *et al.* (1998) Angiogenesis is an early event in the development of chemically induced skin tumors. *Carcinogenesis* 19:2107–13

Brummelkamp TR, Nijman SM, Dirac AM *et al.* (2003) Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. *Nature* 424:797–801

Casanova L, Bravo A, Were F *et al.* (1995) Tissue-specific and efficient expression of the human simple epithelial keratin 8 gene in transgenic mice. *J Cell Sci* 108:811–20

Casanova ML, Bravo A, Martinez-Palacio J *et al.* (2004) Epidermal abnormalities and increased malignancy of skin tumors in human epidermal keratin 8-expressing transgenic mice. *FASEB J* 18:1556–8

Christenson LJ, Borrowman TA, Vachon CM *et al.* (2005) Incidence of basal cell and squamous cell carcinomas in a population younger than 40 years. *JAMA* 294:681–90

De Marval PM, Lutfeali S, Jin JY *et al.* (2011) CYLD inhibits tumorigenesis and metastasis by blocking JNK/AP1 signaling at multiple levels. *Cancer Prev Res (Phila)* 4:851–9

Futscher BW, O'Meara MM, Kim CJ *et al.* (2004) Aberrant methylation of the maspin promoter is an early event in human breast cancer. *Neoplasia* 6:380–9

Gerretsen AL, Beemer FA, Deenstra W *et al.* (1995) Familial cutaneous cylindromas: investigations in five generations of a family. *J Am Acad Dermatol* 33:199–206

Khalkhali-Ellis Z (2006) Maspin: the new frontier. *Clin Cancer Res* 12:7279–83

Kovalenko A, Chable-Bessia C, Cantarella G *et al.* (2003) The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. *Nature* 424:801–5

Lee DA, Grossman ME, Schneiderman P *et al.* (2005) Genetics of skin appendage neoplasms and related syndromes. *J Med Genet* 42:811–9

Luo JL, Tan W, Ricono JM *et al.* (2007) Nuclear cytokine-activated IKKalpha controls prostate cancer metastasis by repressing Maspin. *Nature* 446:690–4

Massoumi R, Chmielarska K, Hennecke K *et al.* (2006) Cyld inhibits tumor cell proliferation by blocking Bcl-3-dependent NF-kappaB signaling. *Cell* 125:665–77

Massoumi R, Paus R (2007) Cylindromatosis and the CYLD gene: new lessons on the molecular principles of epithelial growth control. *Bioessays* 29:1203–14

Moll R, Franke WW, Schiller DL *et al.* (1982) The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* 31:11–24

Moller R, Reymann F, Hou-Jensen K (1979) Metastases in dermatological patients with squamous cell carcinoma. *Arch Dermatol* 115:703–5

Moreno-Maldonado R, Ramirez A, Navarro M *et al.* (2008) IKKalpha enhances human keratinocyte differentiation and determines the histological variant of epidermal squamous cell carcinomas. *Cell Cycle* 7:2021–9

Peinado H, Marin F, Cubillo E *et al.* (2004) Snail and E47 repressors of E-cadherin induce distinct invasive and angiogenic properties in vivo. *J Cell Sci* 117:2827–39

Rajan N, Langtry JA, Ashworth A *et al.* (2009) Tumor mapping in 2 large multigenerational families with CYLD mutations: implications for disease management and tumor induction. *Arch Dermatol* 145:1277–84

Roy HK, Iversen P, Hart J *et al.* (2004) Down-regulation of SNAIL suppresses MIN mouse tumorigenesis: modulation of apoptosis, proliferation, and fractal dimension. *Mol Cancer Ther* 3:1159–65

Saggar S, Chernoff KA, Lodha S *et al.* (2008) CYLD mutations in familial skin appendage tumours. *J Med Genet* 45:298–302

Shi HY, Zhang W, Liang R *et al.* (2001) Blocking tumor growth, invasion, and metastasis by maspin in a syngeneic breast cancer model. *Cancer Res* 61:6945–51

Stark AM, Schem C, Maass N *et al.* (2010) Expression of metastasis suppressor gene maspin is reduced in breast cancer brain metastases and correlates with the estrogen receptor status. *Neurol Res* 32:303–8

Tokuyama R, Satomura K, Maeda E *et al.* (2007) Maspin is involved in bone matrix maturation by enhancing the accumulation of latent TGF-beta. *J Bone Miner Res* 22:1581–91

Trompouki E, Hatzivassiliou E, Tsichritzis T *et al.* (2003) CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. *Nature* 424:793–6

Winter H, Rentrop M, Nischt R *et al.* (1990) Tissue-specific expression of murine keratin K13 in internal stratified squamous epithelia and its aberrant expression during two-stage mouse skin carcinogenesis is associated with the methylation state of a distinct CpG site in the remote 5′-flanking region of the gene. *Differentiation* 43:105–14

Young AL, Kellermayer R, Szigeti R *et al.* (2006) CYLD mutations underlie Brooke-Spiegler, familial cylindromatosis, and multiple familial trichoepithelioma syndromes. *Clin Genet* 70:246–9